UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

TRANSCRIPTIONAL REGULATORY MECHANISMS INVOLVED IN *CYP46A1* UP-REGULATION BY HISTONE DEACETYLASE INHIBITORS

FROM CHROMATIN STRUCTURE TO TRANSCRIPTION FACTORS

Maria João de Jesus Nunes

DOUTORAMENTO EM FARMÁCIA

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Aos meus pais

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ABBREVIATIONS

24OHC	24(S)-hydroxycholesterol
25OHC	25-hydroxycholesterol
27OHC	27-hydroxycholesterol
Aβ	Amyloid-β peptide
ABC	ATP-binding cassette transporter
ACAT	Acyl-CoA:cholesterol acyltransferase
AcH3	Acetylated histone 3
AcH4	Acetylated histone 4
AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
BBB	Blood-brain barrier
CBP	CREB-binding protein
CNS	Central nervous system
CYP7B1	Oxysterol 7α-hydroxylase
CYP27A1	Cholesterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
ChIP	Chromatin immunoprecipitation
DAPA	DNA affinity precipitation assay
DBD	DNA binding domain
DMEM	Dulbecco's modified eagle's medium
DTT	Dithiothreitol
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum

ERK	Extracellular signal-regulated kinase
FPP	Farnesyl pyrophosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGPP	Geranylgeranyl pyrophosphate
H	Histone
HAT	Histone acetyltransferase
HD	Huntington's disease
HDAC	Histone deacetylase
HDACi	HDAC inhibitors
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGCR	HMG-CoA reductase
HMGCS	HMG-CoA synthase
Insig	Insulin-induced gene 1 protein
JNK	c-Jun N-terminal kinase
LAMP-2	Lysosome-associated membrane protein 2
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LTP	Long-term potentiation
LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated kinase kinase
MVK	Mevalonate kinase
NaB	Sodium butirate
NPC	Niemann-Pick type C
NT2	NTERA-2cl.D1
NT2N	NT2 *post-mitotic* neurons
OA	Okadaic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
p-ERK1/2	Phosphorylated form of ERK1/2
p-JNK	Phosphorylated form of JNK
PKA/C	Protein kinase A/C

PP	Protein phosphatase
qPCR	Quantitative real-time PCR
Scap	SREBP cleavage-activating protein
siRNA	Small interfering RNA
SSD	Sterol-sensing domain
RNA pol	RNA polymerase
SAHA	Suberoylanilide hydroxamic acid
SL2	Schneider cell line
Sp	Specificity protein
Sp-RE	Specificity protein-responsive element
SREBP	Sterol regulatory element binding protein
TSA	Trichostatin A
VA	Valproic acid
WB	Western blot

ABSTRACT

Apart from being an essential component of cellular membranes and having a role as a signaling molecule, cholesterol is also the precursor of several bioactive molecules that include bile acids, steroid hormones, oxysterols and vitamin D. In the brain constant levels of this sterol are required for normal functioning and the homeostasis is maintained, in part, by an efficient blood-brain barrier that prevents exchanges with lipoprotein cholesterol from circulation. For that reason, *de novo* and *in situ* synthesis occur to meet cholesterol needs in the central nervous system (CNS), which despite the low synthesis rate must be excreted at some degree in order to keep its steady state. The conversion of cholesterol into 24(S)-hydroxycholesterol, by the neuronal-specific cytochrome P450 cholesterol 24-hydroxylase (CYP46A1) has been described as the major elimination mechanism.

The main goal of this work was to characterize the effect of histone deacetylase (HDAC) inhibition in the transcriptional regulation of *CYP46A1* gene and the molecular mechanism underlying such effect. We started by demonstrating that the inhibition of HDAC activity by trichostatin A (TSA), valproic acid and sodium butyrate cause a potent induction of both *CYP46A1* promoter activity and endogenous expression. Indeed, we have shown for the first time that TSA induces an overall increase in histone acetylation levels at *CYP46A1* proximal promoter, as a result of the detachment of HDACs and recruitment of histone acetyltransferases (HAT), in a process dependent on Sp3 transcription factor decreased binding to particular *cis*-elements. This change in chromatin structure culminates in the recruitment of RNA polymerase II and *CYP46A1* gene activation. Nevertheless, the fact that histone deacetylation was evident at a time point when the HDAC/HAT ratio should still favor acetylation, led us to investigate if mechanisms besides histone hyperacetylation could participate in the TSA mediated derepression of *CYP46A1* gene. Interestingly, we identified the participation of the

mitogen-activated kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling pathway in the *CYP46A1* response to TSA. A decrease in ERK1/2 phosphorylation levels was observed after TSA treatment concomitantly with a decrease in Sp3 binding activity. Inhibition of protein phosphatase activity by pre-treatment with okadaic acid (OA) completely reversed these changes, and impaired the TSA-mediated *CYP46A1* activation without affecting promoter histone hyperacetylation. Our results also show that TSA treatment induces the dissociation of phosphorylated ERK1/2 from the CYP46A1 promoter and specifically from the Sp3-containing DNA fragments. This suggests that in the context of the *CYP46A1* promoter phosphorylated Sp3 acts as a transcriptional repressor being responsible for the recruitment of co-repressor complexes, with and without HDAC activity. Moreover, our work highlights the importance of the MEK-ERK signaling pathway in the control of brain cholesterol elimination.

The importance of CYP46A1 in cholesterol homeostasis and the drastic effect of HDAC inhibitors (HDACi) in its expression, lead us to evaluate if these compounds can affect the expression of other key players in neuronal cholesterol metabolism. In the last part of our work we have identified TSA as a cholesterol-lowering molecule, by modulating the transcription of other genes involved in cholesterol metabolism in human neuroblastoma cells, namely by up-regulating genes that control cholesterol efflux and dow-regulating genes involved in cholesterol synthesis and uptake, thus leading to an overall decrease in total cholesterol content. Moreover, we have shown that TSA is also able to partially reverse the increased cholesterol content and the transcriptional changes, induced by pathological lysosomal accumulation of intracellular cholesterol.

Overall, these results clarify the role of HDACi in the modulation of *CYP46A1* gene transcription as well as other key genes in cholesterol metabolism, comprising a significant contribution in the elucidation of the molecular mechanism involved in the transcriptional regulation of *CYP46A1* gene and emphasizing the idea of HDAC inhibition as a promising therapeutic tool in neurodegenerative disorders with impaired cholesterol metabolism.

Keywords: *CYP46A1* - transcriptional regulation - histone deacetylase inhibition - brain cholesterol metabolism

RESUMO

O colesterol é uma molécula essencial à vida. Para além de desempenhar um papel crucial na estrutura das membranas celulares, através da regulação da permeabilidade e fluidez das mesmas, o colesterol é também percursor de inúmeras moléculas de extrema relevância biológica tais como os ácidos biliares, os oxisteróis, as hormonas esteroides e a vitamina D, podendo também atuar como uma molécula sinalizadora.

No encéfalo, o colesterol está maioritariamente na forma não esterificada e encontra-se associado às bainhas de mielina e às membranas plasmáticas dos neurónios e células da glia. O colesterol é também essencial à formação e propagação de sinapses, ao crescimento dendrítico e à estabilidade dos microtúbulos. Além disso, o sistema nervoso central (SNC) necessita de níveis constantes de colesterol para um correto funcionamento, sendo que a homeostasia é assegurada, em parte, pela barreira hemato-encefálica que impede trocas com o colesterol em circulação. Por esta razão, todo o colesterol presente no encéfalo deriva de uma síntese *de novo* e *in situ*. Apesar de no SNC do adulto a síntese de colesterol ser reduzida, uma fração necessita de ser excretada, de forma a manter os níveis de colesterol constantes. A conversão do colesterol em 24(S)-hidroxicolesterol, pelo enzima 24(S)-hidroxilase (CYP46A1), foi descrita como o principal mecanismo de eliminação. O produto enzimático (oxisterol), contrariamente ao colesterol, atravessa com facilidade a barreira hemato-encefálica, entra em circulação e é posteriormente eliminado no fígado, completando desta forma o processo de transporte reverso do colesterol.

Nos últimos anos, um grande número de estudos aponta para uma relação entre alterações no metabolismo do colesterol e o desenvolvimento de doenças neurodegenerativas, como a doença de Alzheimer, a doença de Huntington ou a doença de Niemann-Pick tipo C. Para além disso, inúmeras evidências sugerem que a indução do

CYP46A1 poderá ter consequências benéficas, nomeadamente na doença de Alzheimer. De facto a sobre-expressão do CYP46A1 poderá ter um efeito inibitório direto sobre a produção do péptido β-amilóide, um efeito indireto relacionado com a diminuição dos níveis de colesterol das membranas neuronais, ou por aumentar a ativação dos genes alvo do receptor nuclear *liver X receptor*.

O principal objectivo deste trabalho foi a caracterização do efeito da inibição das desacetilases de histonas (HDAC) na regulação da transcrição do gene *CYP46A1*, bem como dos mecanismos moleculares subjacentes a esse mesmo efeito. Na primeira parte deste trabalho, começámos por demonstrar que a inibição da atividade das HDACs através do tratamento com os inibidores tricostatina A (TSA), ácido valpróico e butirato de sódio, causam uma drástica indução da atividade do promotor e da expressão endógena do gene *CYP46A1*. Observámos também, pela primeira vez, que o tratamento com TSA induz um aumento significativo dos níveis de acetilação das histonas do promotor próximo do gene *CYP46A1*, sendo que este efeito resulta da diminuição da presença de HDACs e do recrutamento de acetiltransferases de histonas (HAT). Estudos anteriores do nosso grupo identificaram como essenciais na expressão basal do gene *CYP46A1* factores de transcrição da família Sp e, neste estudo, demonstrámos que as proteínas Sp1 e Sp4 são também cruciais para a ativação do *CYP46A1* pelo TSA. No entanto o efeito do TSA parece depender da diminuição da ligação do factor de transcrição Sp3 a elementos de resposta específicos do promotor, uma vez que uma diminuição de ligação desta proteína está relacionada com a diminuição de HDACs presentes no promotor. De uma forma geral, as modificações na estrutura da cromatina e no complexo proteico na região do promotor próximo induzidas pelo TSA culminam no recrutamento da RNA polimerase II e na consequente ativação do gene *CYP46A1*.

A evidência de que o nível de acetilação das histonas a determinado momento não reflete a razão entre os níveis dos enzimas HDAC/HAT presentes no promotor, conduziu-nos à investigação de mecanismos independentes da hiperacetilação de histonas que pudessem contribuir para a ativação do gene *CYP46A1* pelo TSA. De facto, na segunda parte do nosso trabalho identificámos a participação da via de sinalização da cinase de proteínas ativada por mitogénios (MAPK) na resposta do *CYP46A1* ao TSA. Através do pré-tratamento com inibidores químicos específicos bem como a utilização de dominantes negativos da cinase terminal desta via, a cinase de proteínas regulada por sinais extracelulares (ERK), concluímos que a inibição da atividade deste enzima

potencia o efeito do TSA, contrariamente ao efeito da inibição das fosfatases de proteína, através do pré-tratamento com ácido ocadáico, que bloqueia quase na totalidade a ativação do gene *CYP46A1* pelo TSA. A análise dos níveis de fosforilação da ERK1/2 demonstrou que o TSA induz uma diminuição do nível de ativação destas cinases, bem como da atividade de ligação da proteína Sp3 ao DNA, efeitos esses que são revertidos pelo pré-tratamento com ácido ocadáico, sem que o nível de hiperacetilação das histonas do promotor seja afetado. Os nossos resultados demonstram ainda que o tratamento com TSA induz a dissociação da forma fosforilada da ERK1/2 do promotor do *CYP46A1*, especificamente, dos fragmentos de DNA que contêm o factor de transcrição Sp3, sugerindo que no contexto do promotor do *CYP46A1* a proteína Sp3 atua como um repressor da transcrição e é responsável pelo recrutamento de complexos co-repressores, com e/ou sem atividade HDAC. O TSA ao modular a ativação da ERK1/2 regula indiretamente a atividade deste factor de transcrição e consequentemente a expressão do *CYP46A1*. De facto, a replicação do efeito do ácido ocadáico e da inibição da atividade da ERK1/2 na atividade do promotor e expressão basal do gene *CYP46A1* evidencia também a importância desta via de sinalização intracelular no controlo da eliminação de colesterol do encéfalo.

A importância do CYP46A1 na homeostasia do colesterol bem como o efeito drástico dos inibidores das HDACs na sua expressão, conduziu à avaliação do efeito destes compostos na expressão de genes chave no metabolismo do colesterol em células neuronais. Como tal, na última parte deste trabalho demonstrámos a capacidade do TSA de diminuir os níveis de colesterol em células de neuroblastoma humano, através da regulação da expressão de genes envolvidos no metabolismo do colesterol, nomeadamente, através da indução de genes envolvidos no controlo do efluxo (transportadores *ATP-binding cassette*) e catabolismo (CYP46A1), e repressão de genes essenciais para a síntese (3-metilglutaril Coenzima A redutase) e captação de colesterol (receptor de lipoproteínas de baixa densidade), culminado desta forma na diminuição do conteúdo total de colesterol. O tratamento com o composto U18666A, que mimetiza o fenótipo da doença de Niemann-Pick tipo C, caracterizado pela acumulação patológica de colesterol nos lisossomas, resultou no aumento dos níveis de colesterol em células de neuroblastoma humano, bem com no aumento da expressão de genes envolvidos na síntese e captação de colesterol e diminuição dos genes responsáveis pelo efluxo. No entanto, o tratamento com TSA reverteu todos esses efeitos, contribuindo para a correção

das perturbações no metabolismo do colesterol. Apesar de já ter sido demonstrado o efeito benéfico da inibição das HDACs em fibroblastos de pacientes com a doença de Niemann-Pick tipo C, este trabalho demonstra pela primeira vez a capacidade destes compostos de reverter, ao nível da transcrição, os efeitos da acumulação de colesterol nos lisossomas.

Em conclusão, os resultados apresentados nesta tese identificam os inibidores das HDACs como agentes farmacológicos que poderão eventualmente vir a ser usados na indução da transcrição do gene *CYP46A1*, bem como na modelação da transcrição de genes que participam de forma relevante no metabolismo do colesterol no encéfalo. Para além disso constitui uma contribuição significativa para a compreensão dos mecanismos moleculares responsáveis pela regulação da transcrição do gene *CYP46A1*, identificando a via das MAPKs como umas das vias de sinalização responsáveis pelo controlo do catabolismo do colesterol no encéfalo.

A compreensão dos mecanismos moleculares controlados pelos inibidores das HDACs, e envolvidos na homeostasia do colesterol no encéfalo, poderá de facto constituir uma plataforma para o desenvolvimento de possíveis intervenções farmacológicas que vão da neurodegenerescência ao cancro.

Palavras-chave: *CYP46A1* - regulação da transcrição - inibição de desacetilases de histonas - metabolismo do colesterol no encéfalo.

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

Cholesterol belongs to the steroid class of lipids, and is further named sterol due to the modification with a hydroxyl group in the C3 position of its four-ringed hydrocarbon structure (Voet *et al.* 1999). Since it was first isolated in 1784 from the gallstones, cholesterol became the "*most highly decorated small molecule in biology*", as Michael Brown and Joseph Goldstein referred in their Nobel Prize Lecture in 1985 (Brown & Goldstein 1986), reflecting the focus of an incredible number of studies that over the years have highlighted the importance of this molecule and its relevance in both health and disease.

1.1. Cholesterol metabolism

Cholesterol is a multifunctional lipid. As the most abundant single lipid in the membranes of all mammalian cells, cholesterol has an important structural role in membranes by regulating their permeability and fluidity. Cholesterol is also the precursor of several bioactive molecules that include the bile acids, which are synthesized in the liver and help the digestion and absorption of dietary fat, the steroid hormones produced in the endocrine tissues and vitamin D produced in the skin and kidneys (Lodish *et al.* 2004). Other functions of cholesterol include its participation in the processing of the Hedgehog precursor protein, a critical signaling molecule in embryonic development (Incardona & Eaton 2000), and its requirement for proper brain function, which will be later discussed in more detail.

Despite the significant variability in cholesterol content amongst the different cellular membranes (van Meer *et al.* 2008) and between cells from different tissues (Osono *et al.* 1995), cholesterol steady-state levels are critically maintained. The precise control of cholesterol homeostasis in the body is achieved by efficient regulatory mechanisms that coordinate cholesterol synthesis/uptake, storage and efflux.

1.1.1. Cholesterol sources and intracellular cholesterol transport

Animal cells are able to acquire cholesterol from two distinct sources, the endogenous biosynthesis and the exogenous uptake of lipoproteins in circulating plasma. Nevertheless, the predominant source of cholesterol in extra-hepatic tissues is *de novo* biosynthesis (Spady & Dietschy 1983), a mechanism by which cholesterol is synthesized

from acetyl-CoA through the mevalonate pathway (Bloch & Rittenberg 1942, Bloch 1992). Cholesterol synthesis occurs in the endoplasmic reticulum (ER) and cytoplasm in a complex process of approximately thirty enzymatic reactions that is represented in Fig. 1.1.

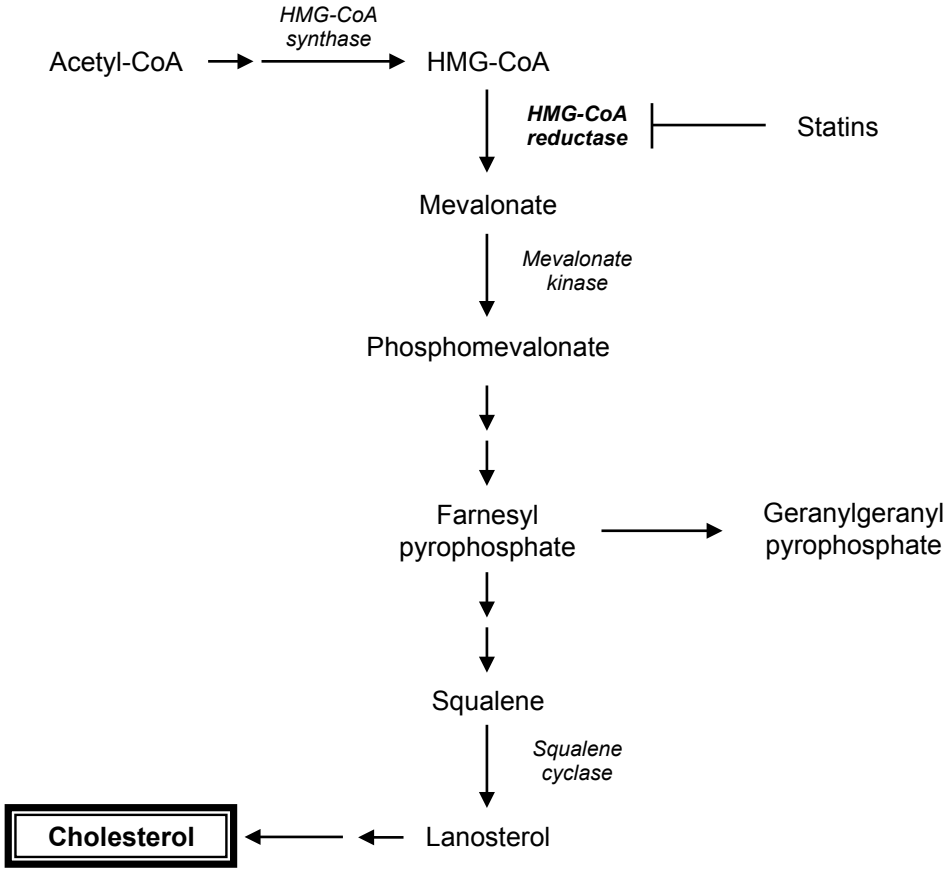

Figure 1.1. The mevalonate pathway of cholesterol biosynthesis. A schematic and condensed representation of cholesterol synthesis from acetyl-CoA through a complex pathway of ≈ 30 reactions. The most relevant enzymes are identified. The 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase catalyzes the rate-limiting step of the pathway, the conversion of HMG-CoA into mevalonate, and is highly regulated at the transcriptional and post-transcriptional level. HMG-CoA reductase is also inhibited by statins, a class of drugs used to lower plasma cholesterol levels. The mevalonate pathway also generates non-sterol isoprenoid intermediates, such as farnesyl and geranylgeranyl pyrophosphate that are precursors of a large group of biologically active molecules.

The first steps in cholesterol biosynthesis occur in the cytoplasm and mediate the formation of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) from acetyl-CoA. The enzyme HMG-CoA synthase (HMGCS) catalyzes the last step of HMG-CoA synthesis.

The conversion of HMG-CoA into mevalonate, the rate-controlling step of the pathway, is catalyzed by HMG-CoA reductase (HMGCR), an integral membrane protein of the ER that is under fine-tune control. This represents one of the mechanisms that contribute to the maintenance of cholesterol homeostasis in the cell. HMGCR is also the target of statins, a class of HMGCR inhibitor drugs that are used to lower plasma cholesterol levels (Jasinska *et al.* 2007). Six subsequent reactions catalyzed by cytosolic enzymes convert mevalonate into squalene. In the first reaction, mevalonate is phosphorylated into phosphomevalonate by the mevalonate kinase (MK) (Hogenboom *et al.* 2004). In those reactions non-sterol isoprenoids are formed that serve as precursors to a large number of biologically active molecules. For instance, the intermediate farnesyl pyrophosphate (FPP) is the precursor of heme *a* and dolichol, whilst ubiquinones, carotenoids and vitamin K and originated from geranylgeranyl pyrophosphate (GGPP) (Nurenberg & Volmer 2012). FPP and GGPP are also substrates for protein isoprenylation, a post-translational modification that mostly occurs in small G proteins such as Ras, Rho and Rac, which confers membrane targeting and attachment (McTaggart 2006). The first committed step for cholesterol biosynthesis is the production of the first sterol in the cascade, lanosterol, from squalene in a reaction catalyzed by squalene cyclase. Finally, the conversion of lanosterol to cholesterol requires a series of reactions that include the loss of three methyl groups and the reduction of double bonds. The enzymes that participate in this final process are embedded in the ER membrane.

Although cholesterol is synthesized in the ER, this organelle contains very low levels of this sterol. After synthesis, cholesterol is targeted primarily to the plasma membrane by non-vesicular mechanism, but also to other sites, such as endosomes. An important mechanism that prevents cholesterol to accumulate in the ER is esterification. Cholesteryl esters are formed by the action of the acyl-CoA:cholesterol acyltransferase (ACAT) and stored in cytosolic lipid droplets (Chang *et al.* 1997). Depending on the cellular cholesterol demands, free cholesterol can in turn be released from lipid droplets by cholesteryl ester hydrolase and directed to other compartments (Ghosh *et al.* 1995, Ghosh 2000, Brown & Goldstein 1983).

Cells can also uptake cholesterol from circulating lipoproteins through the low-density lipoprotein (LDL) receptor (LDLR) pathway. Cholesterol derived from dietary intake is absorbed by the enterocytes of the small intestine and enters circulation in the form of chylomicrons along with triglycerides. These particles are processed and the

cholesterol-enriched remnants are taken up by the hepatocytes. In turn, the liver secretes cholesterol – from chylomicrons remnants or endogenously synthesized – in the form of very low-density lipoproteins. These particles contain triglycerides and cholestryl esters with a shell composed of apolipoproteins (Apo) B/E, a phospholipid monolayer and cholesterol, and are processed in circulation into LDL, which only contains ApoB. The LDL particles are internalized by receptor-mediated endocytosis through the binding to the LDLR. After internalization, in the early endosome, due to the lower pH, the LDLR from the LDL particle can be recycled to the plasma membrane. Cholesteryl esters proceed to late endosomes and are hydrolyzed by acid lipases to form free/unesterified cholesterol. The unesterified cholesterol is subsequently released from late endosomes and delivered to various membranes inside the cell, including the plasma membrane, ER and mitochondria (Ikonen 2008).

1.1.2. Cholesterol efflux and reverse cholesterol transport

Although excess cholesterol can be stored in cytosolic lipid droplets, cells must secrete cholesterol in order to maintain intracellular cholesterol homeostasis. Because most of the extrahepatic cells do not have the ability to catabolize cholesterol, the major mechanism for cholesterol efflux comprises its packaging into high-density lipoproteins (HDL) that are selectively removed from circulation in the liver and steroidogenic cells, and are related to decreased risk of atherosclerotic vascular disease (Rhoads *et al.* 1976). In hepatocytes, cholesterol is secreted in the bile as free cholesterol or after conversion to bile acids; and in steroidogenic cells, it is used for steroid hormone synthesis. The process by which cholesterol is transported from extrahepatic tissues to the liver to be excreted is designated reverse cholesterol transport (Fielding & Fielding 1995). The HDL particles are composed of a cholesteryl esters core, associated with Apo A-I. Selective liver and intestine ATP-binding cassette (ABC) transporter A1 (ABCA1) knockout mice show 80 and 30% reduction in HDL levels, respectively, demonstrating that these organs are major producers of HDL particles. Liver and intestine, that also synthetize Apo A-I are therefore responsible for maintaining HDL levels in a process that is dependent on this membrane efflux transporter (Timmins *et al.* 2005, Brunham *et al.* 2006). ABCA1 promotes cholesterol transfer from the plasma membrane to lipid-poor Apo A-I, leading to the formation of an HDL precursor, the preβ-HDL. In circulation, these particles remove

cholesterol from peripheral tissues and macrophages also in an ABCA1-dependent mechanism. Cholesterol esterification mediated by the lecithin:cholesterol acyl transferase, and further lipidation of the preβ-HDL and cholesterol efflux promoted by the ABC transporters ABCG1, highly expressed in macrophages, and ABCG4, generate the mature HDL particle (Vaughan & Oram 2006, Gelissen *et al.* 2006).

The formation of oxysterols represents another mechanism for cholesterol efflux from cells. Oxysterols are oxidized cholesterol derivatives that are detected in the plasma at very low levels, when compared to cholesterol. Apart from their formation in the early steps of bile acid synthesis from cholesterol in the liver (27- hydroxycholesterol (27OHC) and 7α-hydroxycholesterol), some oxysterols also function as signaling molecules that modulate lipid metabolism. The 27OHC is the most abundant oxysterol in circulation, formed by the action of sterol 27-hydroxylase (CYP27A1) in the mitochondria of macrophages and other extrahepatic cells, and may constitute a mechanism to prevent cholesterol accumulation in tissues (Bjorkhem *et al.* 1994, Lund *et al.* 1996). Moreover, patients with cerebrotendinous xanthomatosis, as a result of mutations in the *CYP27A1* gene, develop premature atherosclerosis (Gallus *et al.* 2006).

1.1.3. Regulation of cholesterol levels

Two different families of transcription factors transcriptionally regulate cholesterol metabolism. The sterol regulatory element binding protein (SREBP) family mediates the increase in cholesterol content, by activating the transcription of genes involved in the synthesis and uptake of cholesterol, and the liver X receptor (LXR) family of nuclear receptors, which by up-regulating genes involved in the efflux of cholesterol from the cell contribute to an overall decrease in cholesterol levels.

The members of the SREBP family of transcription factors include SREBP1, most abundant in the liver and adrenal glands, with the subtypes SREBP1a and 1c generated by alternative splicing, and the ubiquitously expressed SREBP2. SREBP1a is a potent activator of all SREBP-responsive genes, whereas SREBP1c and SREBP2 preferentially activate fatty acid and cholesterol metabolism, respectively (Horton *et al.* 2002). Both SREBP1 and SREBP2 are synthesized as ER precursors, and are subsequently subjected to proteolytic cleavage into their transcriptionally active form.

After maturation in the Golgi, processed SREPBs are translocated to the nucleus where they activate several genes involved in cholesterol, fatty acid and triglyceride metabolism. SREBP2, as the major activator of cholesterol metabolism, regulates the majority of enzymes involved in cholesterol synthesis, including the rate-limiting HMGCR, and also increases the uptake of cholesterol by inducing the LDLR expression (Brown & Goldstein 1999, Horton *et al*. 2002). The SREBPs in the ER interact with the SREBP cleavage-activating protein (Scap), a protein that harbors a sterol-sensing domain (SSD) and functions as a Golgi escort protein (Sakai *et al.* 1997). In low cholesterol conditions, the Scap/SREBP complex leaves the ER in COPII-coated vesicles, which leads SREBP for proteolytic cleavage in the Golgi (Brown & Goldstein 1999, Nohturfft *et al.* 2000). When cholesterol levels rise in the ER, this sterol binds to the SSD of Scap and promotes a conformational change that triggers its association with the insulin-induced gene 1 protein (Insig), an ER retention protein (Adams *et al.* 2003). Insig prevents the binding of Scap to COPII proteins, and retains the SREBPs in the ER membrane (Yang *et al.* 2002, Espenshade *et al.* 2002, Nohturfft *et al*. 2000). The same outcome occurs in the presence of oxysterols, namely 25-hydroxycholesterol (25OHC). However, oxysterols bind to Insig and not to Scap, leading also to the formation of the Insig/Scap/SREBP complex and ER retention (Radhakrishnan *et al.* 2007). Insig-1 is also under a strict regulation at the transcription and protein stability level. When cholesterol levels are high, Scap binds to Insig-1 leading to the stabilization of the protein. When cholesterol levels drop the Scap/SREBP complex dissociates from Insig-1, which is later ubiquitinated and targeted for proteosomal degradation. SREBP, in turn, up-regulates Insig-1 gene and subsequently the protein that will rapidly be degraded until cholesterol levels rise enough to allow its binding to Scap (Gong *et al.* 2006).

The LXRs, which belong to the family of nuclear receptors, also participate in the transcriptional control of cholesterol levels. Both LXRα and LXRβ form a heterodimer with the retinoid X receptor (RXR), another member of the nuclear receptor family, and are activated by sterol metabolites such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol (24OHC), 27OHC and 24(S),25-epoxycholesterol. Moreover, LXRβ is ubiquitously expressed, whereas LXRα is preferentially expressed in tissues involved in lipid metabolism, such as the liver, adipose tissue and macrophages (Repa & Mangelsdorf 2000). LXRs bind to specific DNA sequences and after activation triggers the transcription of the target genes. Activation of LXR induces a decrease in cholesterol

content because it promotes the transcriptional activation of genes involved in cholesterol efflux, such as ABCA1 and G1, and Apo E. Moreover, oxysterol activation of LXRs also induces SREBP1c expression leading to an increase in fatty acid synthesis (Repa *et al.* 2000).

Another classic mechanism for the control of cholesterol levels is the post-transcriptional regulation of HMGCR, which consists in the sterol-induced degradation of the enzyme. HMGCR is present in the ER membrane only for regulatory purposes, although its substrate and product are soluble in water, the membrane-attachment domain was found to be indispensable for the degradation process (Gil *et al.* 1985). This enzyme has a long half-life but when sterols or other isoprenoid derivatives accumulate, HMGCR is rapidly degraded. Both oxysterols (Song & DeBose-Boyd 2004) and the sterol intermediate lanosterol were identified as responsible for this potent feedback response (Song *et al.* 2005). The mechanism of HMGCR degradation is mediated by the ubiquitin-proteasome pathway (Ravid *et al.* 2000) and depends on the ER protein Insig (Sever *et al.* 2003). Lanosterol accumulation promotes the binding of Insig to the SSD of HMGCR, which mediates the ubiquitination of two cytosolic residues and the target of the enzyme to proteasome for degradation. The ubiquitination of HMGCR was recently described to depend on the interplay between two ubiquitin ligases, gp78 and Trc8, and both Insig-1 and Insig-2 in the ER (Jo *et al.* 2011). Furthermore, the enzymatic activity of HMGCR has also been described to be inhibited by AMP-activated protein kinase (AMPK)-dependent phosphorylation (Sato *et al.* 1993, Omkumar *et al.* 1994). Preventing HMGCR phosphorylation, by using a mutant HMGCR, did not affect the posttranscriptional feedback regulation of cholesterol synthesis by mevalonate or oxysterols, although cholesterol synthesis was not suppressed by ATP depletion as observed by *wild type* HMGCR. Since AMPK functions as an energy sensor that is activated by AMP, which is increased when ATP levels decline, it was suggested that the AMPK-dependent phosphorylation occurred as a response mechanism to decreased energy stores in the cell (Sato *et al*. 1993).

More recently, the participation of microRNA (miRNA)-mediated regulation of cholesterol metabolism was also described. miR-33a and miR-33b, intronic miRNAs located in the SREBP2 and SREBP1 genes, respectively, were identified to target ABCA1, ABCG1 and Niemann-Pick type C (NPC) 1 (Najafi-Shoushtari *et al.* 2010, Rayner *et al.* 2010, Marquart *et al.* 2010), and cooperate with SREBP-dependent

mechanism to increase intracellular cholesterol levels, in cholesterol poor conditions. Interestingly, inhibition of miR-33 increased HDL levels and reverse cholesterol transport in several animal models (Rayner *et al.* 2011b, Rayner *et al.* 2011a), concomitantly with a regression of atherosclerosis (Rayner *et al*. 2011b).

1.2. Cholesterol metabolism in the brain

Cholesterol metabolism in the brain has a particular difference compared to most of other extra-hepatic tissues: the presence of the blood-brain barrier (BBB). This is a highly specialized brain endothelial structure of the fully differentiated neurovascular system, which limits the entry of plasma components, red blood cells, leukocytes, and also all the large molecule neurotherapeutics (e.g. recombinant peptides, anti-sense agents and genetic vectors) and the majority of small molecule drugs, from the circulation into the brain (Zlokovic 2008). For this reason the brain has no access to lipoprotein particles in the plasma and, therefore, must synthesize cholesterol *de novo* and *in situ* to account for all the cholesterol needs in this organ. Since the early 1940s, studies performed with different animal models and also in humans suggested the absence of interchangeability of cholesterol between brain and circulation (Waelsch *et al.* 1940b, Bloch *et al.* 1943, Chobanian & Hollander 1962). Further studies confirmed that the majority of brain cholesterol content was *de novo* synthesized both during early development and in adult animals (Waelsch *et al.* 1940a, Jurevics & Morell 1995, Turley *et al.* 1998, Meaney *et al.* 2001, Quan *et al.* 2003).

As the most cholesterol-rich organ, the brain contains approximately a quarter of total cholesterol content in the human body. The cholesterol present in the central nervous system (CNS) is essentially unesterified and distributed in two major pools: the membranes of myelin and the plasma membranes of neurons and glial cells (Dietschy & Turley 2004). Myelin is a specialized membrane produced by the oligodendrocytes that provides electrical insulation around the axon, ensuring a rapid propagation of action potentials. Contrary to what is found in other plasma membranes, where cholesterol content represents less than 20% of its dry weight, the myelin membrane contains 26% of cholesterol and an overall 70% of lipids (Chrast *et al.* 2011). Apart from being an important structural component of the CNS, cholesterol is also essential for myelination

during brain maturation (Saher *et al.* 2005), modulation of dendrite outgrowth and microtubule stability (Fan *et al.* 2002), and synaptogenesis (Mauch *et al.* 2001, Goritz *et al.* 2005).

In both mouse and human brains the total cholesterol content dramatically increases after birth, reflecting the process of myelin assembly that occurs in the first month and during the first two years of postnatal life, respectively, and accumulates to constant values in the adult state (Chrast *et al.* 2011, Dietschy & Turley 2004). Furthermore, by measuring the rate of cholesterol synthesis in mice brains, Quan and co-workers (Quan *et al.* 2003) demonstrated that it is not constant during the different stages of development. In the first three weeks, a peak of cholesterol synthesis occurs, directly reflecting the rate of cholesterol accumulation, as a consequence of the myelination process, and gradually decreases to a constant level of approximately 0.035 mg/day as the animal ages. Concomitantly, in the adult animal, the rate of accumulation also decreases to 0.012 mg/day. Since brain cholesterol content is kept constant in the adult state, and due to the fact that at the same time the rate of cholesterol synthesis is higher than the rate of cholesterol accumulation, a mechanism for cholesterol excretion of 0.023 mg/day across the BBB must take place. Although brain cholesterol is efficiently reutilized, resulting in a long half-life (Bjorkhem *et al.* 1998), a dynamic cholesterol turnover occurs also with very precise regulatory mechanisms that ensure the homeostasis required for proper brain function.

1.2.1. Brain cholesterol synthesis, transport and efflux

The brain is composed of neurons, which are cells specialized in the generation and transmission of electrical signals, and glial cells - oligodendrocytes, astrocytes and microglia - that actively support and assist neurons in several functions, such as synaptogenesis (Eroglu & Barres 2010). Cholesterol in the CNS is mainly unesterified and is present in the myelin sheets and in the plasma membranes of neurons and glial cells (Dietschy & Turley 2004). During development, cholesterol synthesis is very high and mainly associated to myelination by oligodendrocytes (Quan *et al.* 2003). In the mature brain, this rate drastically decreases and reflects the synthesis in neurons and glial cells (Dietschy & Turley 2004). However, it has not yet been clarified *in vivo* the exact extent of cholesterol synthesis in those cells. The current view is that from the embryonic

to the adult stage, neurons partially abandon cholesterol synthesis, relying on astrocyte production and delivery via ApoE containing lipoparticles (Pfrieger 2003, Nieweg *et al.* 2009). The explanation proposed is that neurons specialize on electrical conduction and reduce the energy-consuming task of cholesterol biosynthesis. In fact, it has been demonstrated that neuronal precursors must synthesize cholesterol to survive (Saito *et al.* 2009), but the same is not true for adult neurons (Funfschilling *et al.* 2007), which apparently can meet their cholesterol needs from other sources. Moreover, *in vitro* studies demonstrated that neurons when compared to glial cells produce cholesterol at a lower rate and have a distinct profile of cholesterol biosynthetic enzymes, precursors and metabolites. Interestingly, in the presence of glial cells neurons reduce cholesterol synthesis to about 20% (Nieweg *et al.* 2009). Nevertheless, several studies support the fact that some adult neurons retain the ability to synthesize cholesterol. HMGCR and 7-dehydrocholesterol reductase, the last enzyme of the mevalonate pathway, have been described to be widely expressed in neurons throughout adult murine brain (Korade *et al.* 2007). SREBP2 is expressed particularly in pyramidal neurons of the hippocampus (Kim & Ong 2009). Moreover, Valdez and co-workers demonstrated that neurons are able to synthesize cholesterol in a study where all cholesterol metabolism markers were detected in hippocampal neurons, suggesting that this sterol is indeed locally produced (Valdez *et al.* 2010).

The importance of astrocytes as the cholesterol suppliers in the brain was outlined in studies aiming to identify the role of glial cells in synaptogenesis. Pfrieger and co-workers observed that neurons require glial cell for efficient synaptic activity (Pfrieger & Barres 1997), which later was identified as Apo E containing lipoparticles that were produced by glial cells (Mauch *et al.* 2001). Furthermore, Apo E, the major apolipoprotein in the CNS, is mainly synthesized by astrocytes in HDL-like lipoparticles, although it has also been detected in neurons after ischemia or oxidative stress (Aoki *et al.* 2003), and Apo J is also abundant (DeMattos *et al.* 2001). LDLR family members are expressed in the CNS, including neurons, and the Apo E containing lipoproteins from glial cells were also described to stimulate axonal growth of neurons through the LDLR family (Hayashi *et al.* 2004). The shuttle of cholesterol from astrocytes to neurons (Fig. 1.2) is therefore mediated by Apo E lipoparticles that are internalized in neurons by the LDLR. The LDL-related protein 1 (LRP1), which is expressed in neurons (Bu *et al.* 1994), appears to be also crucial for cholesterol transport (Liu *et al.* 2010).

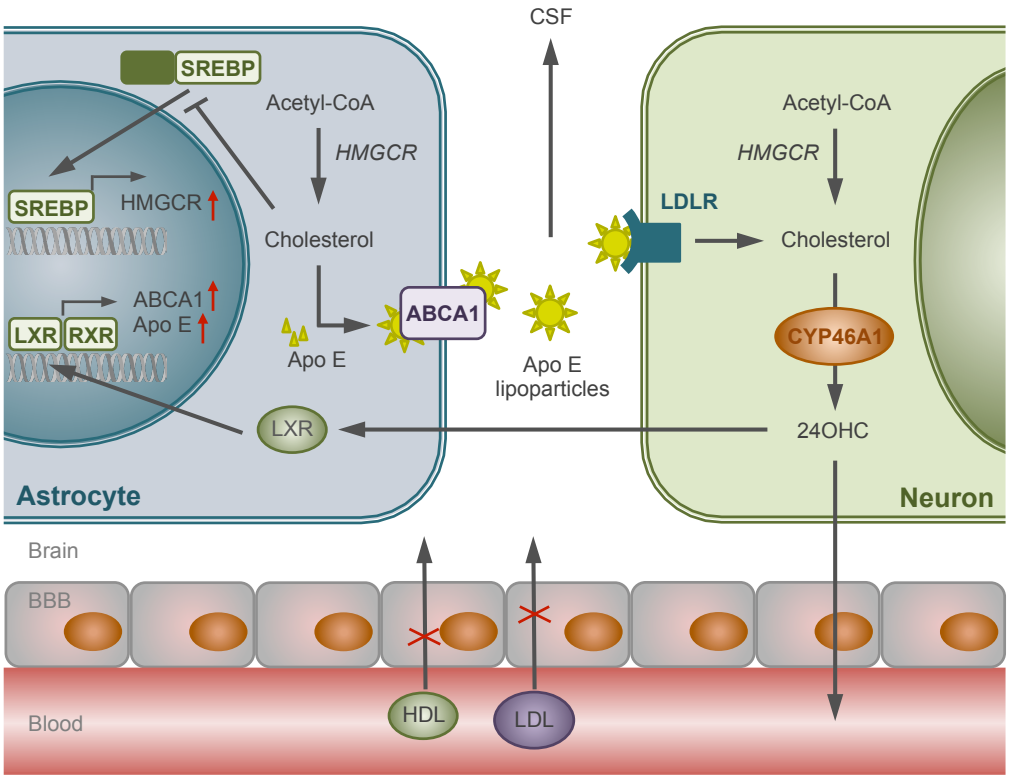

Figure 1.2. Cholesterol metabolism in the CNS. In the adult brain astrocytes produce the majority of cholesterol, which is secreted through ABCA1 in Apo E containing lipoproteins. Neurons internalize these particles by endocytosis in a LDL receptor-mediated pathway, and cholesterol is distributed inside the cell, where it has several important biological functions. Excess cholesterol is converted into 24(S)-hydroxycholesterol (24OHC) by the neuronal-specific cytochrome P450 CYP46A1, and represents the major mechanism for brain cholesterol turnover. 24OHC is also a signaling molecule that control cholesterol homeostasis in the brain. CYP46A1 - cholesterol 24-hydroxylase. *Adapted from Karasinska & Hayden (2011).*

In the brain, ABC transporters are also essential for Apo E-containing lipoprotein formation. ABCA1, ABCG1 and ABCG4 are highly expressed in the brain, both in neurons and glial cells (Hirsch-Reinshagen *et al.* 2004, Tachikawa *et al.* 2005). The specific loss of ABCA1 in neurons and glial cells reduced brain cholesterol, decreased synapse number and increased the uptake of cholesterol from plasma HDL (Karasinska *et al.* 2009), suggesting that ABCA1 is essential for the formation of Apo E lipoparticles. Knockout mice studies for ABCG1 and ABCG4 also demonstrated the participation of these transporters in HDL particle formation in the CNS, and possibly in a recycling process of cholesterol and other sterols (Wang *et al.* 2008a).

As mentioned before, in the adult brain a mechanism of cholesterol elimination must exist to compensate for the synthesis, and maintain the homeostasis (Quan *et al.* 2003). The major mechanism was identified as the conversion of cholesterol into 24OHC (Lutjohann *et al.* 1996, Bjorkhem *et al.* 1998, Xie *et al.* 2003). The hydroxylation of cholesterol is catalyzed by cholesterol 24-hydroxylase (CYP46A1), a neuronal-specific enzyme that belongs to the cytochrome P450 superfamily (Lund *et al.* 1999). This oxysterol, contrary to cholesterol, can pass the BBB and enter circulation, where it associates with LDL and HDL particles (Babiker & Diczfalusy 1998), being degraded in the liver by conversion to bile acids and subsequently eliminated (Bjorkhem *et al.* 2001). Although identified in the human brain in the 1970s, only in the 1990s Bjorkhem and co-workers determined that more than 90% of the circulating levels of 24OHC were originated from the brain (Lutjohann *et al.* 1996). As a potent LXR activator, 24OHC regulates cholesterol metabolism in the CNS. *In vitro* the release of this oxysterol by neuronal cells induces the secretion of Apo E lipoproteins by astrocytes, through the activation of Apo E and ABCA1 genes in those cells (Abildayeva *et al.* 2006).

In summary, glial cells produce cholesterol that is packaged into Apo E lipoparticles, with the participation of the ABC transporters. In neurons those particles are internalized trough the LDLR pathway and cholesterol is distributed inside the cell. The formation of 24OHC by CYP46A1 mediates cholesterol turnover and excretion of excess cholesterol. Cholesterol turnover in neurons promote the efflux of cholesterol from astrocytes, in a 24OHC-dependent activation of the LXR pathway (Fig. 1.2).

1.2.2. Cholesterol in neurodegenerative disorders

Apart from being a fundamental component of all cells, cholesterol is also a molecule with diverse biological functions, essential for proper growth and development. Disorders with impaired cholesterol synthesis that are compatible to life are associated with malformation syndromes. The Smith-Lemli-Opitz syndrome is one of the most common, and is caused by a deficiency of 7-dehydrocholesterol reductase activity, the enzyme that catalyzes the last step of cholesterol synthesis (Porter & Herman 2011). Moreover, due to the importance of cholesterol in neuronal structure and activity, and the need of constant levels of this sterol for normal brain function, it is not surprising that a

number of diseases with defects in cholesterol metabolism and transport are associated with neurological symptoms.

1.2.2.1. Alzheimer's disease

As the most prevalent cause of dementia worldwide, Alzheimer's disease (AD) is an age-associated progressive neurodegenerative disorder that results in cognitive impairment and memory deterioration. This disease primarily affects the brain areas responsible for learning and memory (cortex and hippocampus) and is associated with neurotoxicity, synaptic failure and progressive neuronal loss (Querfurth & LaFerla 2010). The hallmarks of AD are the deposition of extracellular amyloid-β peptide (Aβ) in neuritic plaques, as a result of the cleavage of amyloid precursor protein (APP) by β-secretase, and the presence of intracellular neurofibrillary tangles in pyramidal neurons that are composed of hyperphosphorylated and aggregated form of tau protein. Evidence suggests a link between cholesterol metabolism and the onset of AD. As mentioned above, Apo E as the major lipoprotein in the brain has a fundamental role in lipid transport and distribution, and Apo E containing lipoparticles are essential for synaptogenesis, dendritic growth and neuronal repair (Hayashi 2011). In humans, the Apo E isoforms, Apo E2, Apo E3 and Apo E4 are encoded by the alleles ε2, ε3 and ε4, respectively (Mahley 1988). The Apo E ε4 allele is the major genetic risk factor for AD as the number of ε4 allele correlates with increased risk and decreased age of disease onset (Strittmatter & Roses 1996), and with increased neuritic plaques in the brain (Tiraboschi *et al.* 2004), suggesting that the Apo E4 isoform accelerates Aβ deposition. In fact, it has been recently demonstrated an association between Apo E ε4 gene and fibrillar Aβ burden in the brain of cognitively normal subjects (Reiman *et al.* 2009). Additionally, Apo E2 and Apo E3 complexes with Aβ are cleared at the BBB at a faster rate than Apo E4 complexes, suggesting that the correlation between Apo E isoforms and AD could be associated with the differential efficiency of Aβ clearance from the brain (Deane *et al.* 2008). Other roles of Apo E4 in AD have also been suggested, including detrimental effects on neuronal plasticity, stimulation of tau phosphorylation, and disruption of the cytoskeleton and impairment of mitochondrial function (Huang 2010). Recently, by using different Apo E transgenic mice, Bell and collaborators (Bell *et al.* 2012) have shown that

expression of ApoE4 but not ApoE2 and ApoE3, leads to BBB breakdown by activating proinflammatory pathways in pericytes. This, in turn, leads to neuronal uptake of multiple blood-derived neurotoxic proteins, and microvascular and cerebral blood flow reductions. Interestingly, it was recently shown that Apo E-directed therapeutics rapidly clear Aβ and reverse deficits in AD mouse models (Cramer *et al.* 2012).

Moreover, cholesterol-rich membranes seem to favor the cleavage of APP, through the amyloidogenic pathway into Aβ (Simons *et al.* 1998) (Fig. 1.3). In addition, *in vitro* and *in vivo* studies suggested that inhibiting cholesterol production reduces Aβ levels (Fassbender *et al.* 2001) and promotes the APP processing through the non-amyloidogenic pathway (Kojro *et al.* 2001).

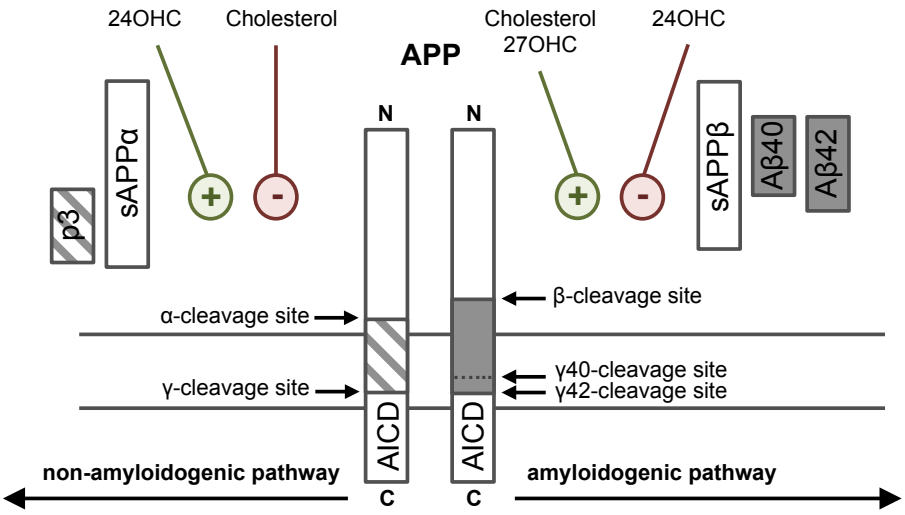

Figure 1.3. Cholesterol and oxysterols in APP processing. The amyloid precursor protein (APP) can be sequentially cleaved by α- and γ-secretase or β- and γ-secretase, through the non-amyloidogenic or amyloidogenic pathway, respectively. The amyloidogenic pathway leads to extracellular deposition of Aβ, one of the hallmarks of AD. Both cholesterol and oxysterols appear to modulate this protein processing, as indicated by the positive and negative signs. *Adapted from Grosgen et al. (2010).*

Hypercholesterolemia has also been established as a risk factor for AD. In autopsy studies, a connection has been determined between high cholesterol levels in plasma and the presence of AD pathology markers in brains (Launer *et al.* 2001, Pappolla *et al.* 2003). Because brain cholesterol is independent from plasma cholesterol, it has been suggested that the flux of 27OHC across the BBB could be the link (Bjorkhem *et al.* 2009). Firstly, cholesterol and 27OHC levels in circulation significantly correlate (Harik-

Khan & Holmes 1990, Babiker *et al.* 2005); it is possible that higher uptake of 27OHC to the brain occurs under hypercholesterolemia conditions. Secondly, 27OHC has been described to favor the amyloidogenic pathway of APP processing in human neuroblastoma cells (Prasanthi *et al.* 2009) (Fig. 1.3), and to down-regulate the activity-regulated cytoskeleton-associated (Arc) protein in hippocampal neurons, which recapitulated the *in vivo* effects of high fat diet induce hypercholesterolemia in rodent brain (Mateos *et al.* 2009). Arc is a key molecule for maintenance of synaptic potentiation and long-term consolidation of memory shown to be decreased in AD brains (Guzowski *et al.* 2000, Ginsberg *et al.* 2000).

In accordance, cholesterol-lowering drugs such as statins have also been proposed to have a beneficial outcome in AD patients and although some epidemiological studies revealed that the prevalence of AD is reduced in patients treated with statins (Wolozin *et al.* 2000, Jick *et al.* 2000), the epidemiologic studies have highly variable outcomes (Shepardson *et al.* 2011). The protective effects of statins in AD are controversial but a recent study concluded that there is still no evidence from randomized controlled trials that statins are beneficial in treatment of dementia (McGuinness *et al.* 2012).

1.2.2.2. Niemann-Pick type C disease

Dysfunctional cholesterol metabolism can also be found in the Niemann-Pick type C disease (NPC), a lethal and autosomal-recessive lipid storage disorder, characterized by intracellular accumulation of unesterified cholesterol and glycosphingolipids in late-endosomal/early lysosomal compartments (Vanier & Millat 2003). Cholesterol accumulation can be observed in neurons of the cerebral cortex, cerebellum and hippocampus (Paul *et al.* 2004), and also in non-neuronal tissues (Beltroy *et al.* 2005). Clinically, NPC disease manifests as a hepatic and neurodegenerative disorder, with neurological symptoms including cerebellar ataxia, dysarthria, dysphagia, and progressive dementia, leading to premature death. This disease is a consequence of mutations in the genes encoding for NPC1 (95%) or NPC2 (5%) proteins, which are involved in intracellular cholesterol trafficking (Vanier & Millat 2003). Since NPC1 and NPC2 proteins appear to bind cholesterol in opposite orientation, a model has been proposed where unesterified cholesterol binds to NPC2, a soluble protein of late

endosome/lysosome, and is subsequently transferred to the N-terminal cholesterol binding domain of NPC1, anchored in the membrane of these organelles. The mechanism by which cholesterol is released from late-endosomes to plasma membrane and ER is currently unknown (Peake & Vance 2010). It also appears that only the trafficking of LDL-derived cholesterol is defective in NPC model, whereas endogenous synthesis and HDL-derived cholesterol are essentially unaffected (Shamburek *et al.* 1997, Xie *et al.* 2000). Nevertheless, it has also been described that even newly synthesized cholesterol accumulates in intracellular compartments after reaching the plasma membrane, having the same fate of LDL-derived cholesterol, although in a much slower process (Cruz & Chang 2000). The absence of unesterifed cholesterol reaching the ER fails to control cholesterol homeostasis leading to a defective suppression of cholesterol synthesis and uptake, (Liscum & Faust 1987), concomitantly with impaired cholesterol esterification in the ER (Pentchev *et al.* 1985). Moreover, LXR-mediated gene expression, which mediates cholesterol efflux, is attenuated in NPC mutants as well as the production of oxysterol stimulated by LDL cholesterol. Treatment with exogenous oxysterols (25OHC and 27OHC) was able to decrease cholesterol content and to revert the NPC phenotype in mutant fibroblasts (Frolov *et al.* 2003). Interestingly, LXR activation in $Npc1^{-/-}$ mice increased cholesterol excretion from the brain, decreased neuroinflammation and extended the lifespan (Repa *et al.* 2007). Despite cholesterol accumulation in both $Npc1^{-/-}$ glia and neurons, the secretion of Apo E-containing lipoproteins from astrocytes does not depend on NPC1 protein (Karten *et al.* 2005), and NPC1 deficient neurons accumulate cholesterol in the cell body, independently of exogenously supplied cholesterol. Nevertheless, cholesterol content of distal axons decreases, suggesting that trafficking of endogenously synthesized cholesterol in $Npc1^{-/-}$ neurons is impaired (Karten *et al.* 2002). On the other hand, restoring the Npc1 in $Npc^{-/-}$ mice only in astrocytes enhanced survival and decreased neuronal storage of cholesterol and neuronal degeneration (Zhang *et al.* 2008). Despite the increasing knowledge regarding cholesterol trafficking in the CNS, the mechanism by which cholesterol accumulation leads to neurodegeneration remains poorly defined.

1.2.2.3. Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor disturbances and cognitive decline caused by a mutation in the huntingtin gene that leads to an expansion of a polyglutamine tract. Although mutant huntingtin is ubiquitously expressed, cellular degeneration occurs mainly in the brain. In the HD brain, the striatum and cerebral cortex are the most affected regions, with an altered intracellular localization and perinuclear accumulation of mutant huntingtin, resulting in the formation of neuronal intranuclear inclusions and protein aggregates in cytoplasm, dendrites and axon terminals (Ross & Tabrizi 2011). Nevertheless, accumulating evidence indicates that *wild type* huntingtin may exert a variety of intracellular functions from protein trafficking, vesicle transport and anchoring to the cytoskeleton, and clathrin mediated endocytosis to postsynaptic signaling, transcriptional regulation and anti-apoptotic function (Gil & Rego 2008).

In 2002, a study from Elena Cattaneo's laboratory, where transcriptional profile in striatal cells expressing mutant huntingtin was assessed by microarray analysis suggested the involvement of cholesterol metabolism in early HD pathogenesis (Sipione *et al.* 2002). Later, it was reported that the mRNA levels of the cholesterogenic pathway genes were severely reduced *in vitro* and *in vivo* models of HD, probably due to the reduction of 50% in the nuclear active form of SREBP, as well as in human *postmortem* brain tissues (Valenza *et al.* 2005). Moreover, in the brain of R6/2 huntingtin-fragment mice model, it was observed a progressive decrease of cholesterol precursors levels, namely lathosterol and lanosterol, and a reduction of HMGCR activity from pre-symptomatic stages. This reflects the decrease in the cholesterol biosynthetic pathway in HD brains, although the steady-state levels of cholesterol remain unchanged (Valenza *et al.* 2007b). These results were also confirmed in the brain of the yeast artificial chromosome (YAC128) mouse model of HD, which expresses the full-length mutant human huntingtin, despite the absence of any change in sterol content or HMGCR activity in the liver, suggesting that a compensatory mechanism might occur in this organ. Interestingly, lower brain and plasma 24OHC levels were observed in both mice models of HD (Valenza *et al.* 2007a, Valenza *et al.* 2007b). In human HD patients a significant decrease in 24OHC levels was also observed, probably reflecting the neuronal loss that characterizes this pathology, suggesting that 24OHC levels can complement the analysis

of the neurodegenerative changes observed in the early stages of HD (Leoni *et al.* 2008). More recently, the levels of lanosterol and lathosterol in blood, as well as that of 27OHC were significantly reduced, which suggests that whole-body and brain cholesterol homeostasis are impaired in HD patients (Leoni *et al.* 2011). A hypothesis is that a decrease in the levels of 24OHC could reduce the signaling of LXR-mediated transcription toward astrocytic production and efflux of cholesterol. In fact, primary astrocytes from YAC128 mice model have reduced mRNA levels of ABCA1, ABCG4 and Apo E compared with controls, which led to reduced production and secretion of Apo E. In the cerebrospinal fluid of these mice Apo E was also associated with smaller lipoproteins, indicating a reduction in cholesterol transport between astrocytes and neurons in the HD brain (Valenza *et al.* 2010). In the same study, the R6/2 model also displayed a lower sterol content in synaptosomes, which may contribute to the synaptic dysfunction described in HD models, and myelin in symptomatic stages. These evidences point to an association between HD and decreased cholesterol biosynthesis, although it remains to be answered if it is a cause or a consequence of the disease.

1.3. Cholesterol 24-hydroxylase - CYP46A1

1.3.1. Gene expression and protein function

Cholesterol 24-hydroxylase (CYP46A1) belongs to the cytochrome P450 superfamily and is responsible for the conversion of cholesterol into 24OHC, by catalyzing the addition of a hydroxyl group to carbon 24 of the cholesterol molecule (Lund *et al.* 1999). The human *CYP46A1* gene is located on chromosome 14q32, contains 15 exons, and encodes for a protein of approximately 500 amino acids. The human and mouse CYP46A1 mRNA orthologues are 95% identical in sequence, in contrast with the average 70% observed with other cytochromes P450 (Lund *et al.* 1999). The sequence conservation is also present throughout the vertebrates, which underlies the importance of CYP46A1 and cholesterol turnover in the brain. CYP46A1 is responsible for the conversion of cholesterol into 24OHC, and catalyzes the metabolism of 24OHC into 24,25- and 24,27-dihydroxycholesterols (Mast *et al.* 2003).

Unlike other cytochromes P450 that are preferentially expressed in the liver (Xie *et al.* 2003), CYP46A1 expression was largely confined to the brain. In both humans and mice, CYP46A1 is detectable after birth (Lund *et al.* 1999), although in mice is already present in some neurons of the developing embryo (Lund *et al.* 2003). In mice, Cyp46a1 protein levels significantly increase after myelination concomitantly with a peak in 24OHC levels, a phase where cholesterol synthesis rapidly declines. Both CYP46A1 and 24OHC levels are maintained constant during adulthood (Lund *et al.* 1999). In mouse, low levels of CYP46A1 mRNA can also be detected in liver and testis. More extensive tissue surveys confirmed the preferential expression of the mRNA in the human brain (Nishimura *et al.* 2003). More recently, *in situ* hybridization and immunohistochemical experiments indicated that CYP46A1 mRNA and protein are present in neurons and not in support cells. CYP46A1 is localized in the ER and is distributed throughout the cell bodies and dendrites of multiple types of neurons (Ramirez *et al.* 2008). Expressing cells include large pyramidal neurons in various layers of the cortex and the hippocampus, as well as Purkinje cells in the cerebellum (Lund *et al.* 1999, Ramirez *et al.* 2008). Additionally, expression of 24-hydroxylase was found in hippocampal and cerebellar interneurons, in retinal ganglion cells, and in a subset of retinal cells localized in the inner nuclear layer (Ramirez *et al.* 2008, Bretillon *et al.* 2007).

Transcriptional regulation studies of *CYP46A1* revealed that the promoter has no canonical TATA or CAAT boxes and has a high GC content, a feature often found in genes considered to have a largely housekeeping function (Ohyama *et al.* 2006, Milagre *et al.* 2008). No substrate-dependent transcriptional regulation was observed, and treatment of neuroblastoma cells with a broad spectrum of endogenous and exogenous compounds did not result in any significant change in *CYP46A1* promoter activity, although it was suggested that oxidative stress slightly increased *CYP46A1* expression (Ohyama *et al.* 2006). Moreover, our group demonstrated the participation of the Sp family of transcription factors in *CYP46A1* brain specific expression, with an increase in the ratio of (Sp3+Sp4)/Sp1 proteins bound to the proximal promoter Sp-response elements *in vitro*, when using nuclear extracts prepared from primary rat cortical neurons (Milagre *et al.* 2008). Chromatin immunoprecipitation (ChIP) analysis confirmed the association of these transcription factors to the *CYP46A1* promoter in human *post-mitotic* neurons. A decrease in Sp1 binding during differentiation of NT2 teratocarcinoma cells into *post-mitotic* neurons was observed, concomitantly with a significant increase in

CYP46A1 mRNA levels (Milagre *et al.* 2012a). Interestingly, increased CYP46A1 protein levels and decreased levels of CYP27A1 detected during the differentiation process suggests that these cells shift from CYP27A1 to CYP46A1 cholesterol catabolic pathway with neuronal commitment, despite no enzyme-product correlations were observed (Milagre *et al.* 2012b).

Chromatin modifying agents have also been described to modulate *CYP46A1* expression (Shafaati *et al.* 2009, Milagre *et al.* 2010). Histone deacetylase inhibitors (HDACi) significantly increase CYP46A1 mRNA levels *in vitro* and in *vivo* (Shafaati *et al.* 2009). We have also demonstrated that pre-treatment of human neuroblastoma cells with the demethylating agent 5-aza-deoxycytidine significantly potentiates the HDACi-mediated activation of CYP46A1 expression, in a DNA methylation independent mechanism, by decreasing Sp3 recruitment to the proximal promoter, as well as histone deacetylase (HDAC) 1 and 2 (Milagre *et al.* 2010). These enzymes are involved in chromatin remodeling by promoting histone deacetylation, a histone mark usually associated with gene repression, and can be recruited to promoters by Sp transcription factors. More recently, the antifungal drug voriconazole was shown to inhibit CYP46A1 *in vitro* and *in vivo*, and reduce levels of 24OHC, cholesterol precursors and HMGCR mRNA (Shafaati *et al.* 2010).

Cyp46a1 knockout mice were generated by David Russell's laboratory (Lund *et al.* 2003). The animals develop normally and the whole-body fatty acid and cholesterol metabolism are also normal. Although plasma 24OHC levels were decreased by 60%, no effect in lipid metabolism in non-brain tissues was observed, suggesting that Cyp46a1 has almost no contribution in peripheral tissues. However, in the brain, the inactivation of Cyp46a1 resulted in a decrease of 40% in cholesterol synthesis, and despite the reduction in cholesterol turnover the amount of cholesterol in this tissue remained unchanged. This observation suggests that cholesterol accumulation due to the absence of the Cyp46a1 catabolic pathway suppresses further synthesis, in a negative feedback loop, resulting in the maintenance of steady state cholesterol levels. Furthermore, the loss of cholesterol 24-hydroxylase reduced the excretion of sterols from the brain but did not abolished this removal process, indicating that there must be other pathways by which cholesterol is eliminated from this tissue (Lund *et al.* 2003).

Transgenic mice overexpressing Cyp46a1 have increased levels of 24OHC in brain and plasma, but not in the liver (Shafaati *et al.* 2011b). Although it has been

described that 24OHC and other LXR agonists induce astrocyte-mediated efflux of Apo E-containing lipoparticles as well as Apo E and ABCA1 mRNA levels (Abildayeva *et al.* 2006), in Cyp46a1 transgenic mice no significant changes were observed in LXR-dependent gene expression despite the observed increase in 24OHC levels. Since the 24OHC effect in cholesterol efflux appears to be directed to astrocytes and not to neurons (Abildayeva *et al.* 2006), we cannot exclude the possibility that the use of whole brain homogenates for the expression studies in Cyp46a1 transgenic mice could be masking such changes (Shafaati *et al.* 2011b). Nevertheless, in accordance to what was previously observed in *Cyp46a1*$^{-/-}$ mice, increased levels of cholesterol precursors were detected in transgenic mice, which compensate the increase in cholesterol turnover (Shafaati *et al.* 2011b).

1.3.2. CYP46A1, cholesterol turnover and learning

Behavioral tests performed in *Cyp46a1*$^{-/-}$ mice underscored an important role of both CYP46A1 and cholesterol turnover in higher order brain function, such as learning and memory (Kotti *et al.* 2006, Kotti *et al.* 2008).

As assessed by three types of behavioral learning assays cholesterol 24-hydroxylase knockout mice exhibited severe deficiencies in spatial, associative and motor learning (Kotti *et al.* 2006). Usually these profound disabilities are associated with severe defects in some brain regions such as the hippocampus. However, histological studies did not reveal any obvious anatomical shortcomings in *Cyp46a1*$^{-/-}$, which suggests that the learning defects could be due to metabolic abnormalities in the animals (Kotti *et al.* 2006). Indeed, measurement of long-term potentiation (LTP) in hippocampal slices from *wild type* and *Cyp46a1*$^{-/-}$ mice revealed that LTP is impaired in the absence of Cyp46a1. Interestingly, treatment of *wild type* hippocampal slices with a statin reproduced the LTP reduction observed in knockout slices, suggesting that cholesterol synthesis is important for LTP. In fact, in *wild type* slices treated with statin, a supplementation with mevalonate but not cholesterol was able to restore LTP, which clearly indicates that cholesterol synthesis must occur. In knockout slices both mevalonate and geranylgeraniol, a nonsterol isoprenoid produced by the mevalonate pathway were able to reverse the LTP blockage (Kotti *et al.* 2006, Kotti *et al.* 2008). The mechanism by which geranylgeraniol facilitates LTP is currently unknown.

With these studies Kotti and co-workers concluded that cholesterol turnover in brain mediated by Cyp46a1, activates the mevalonate pathway and that a constant production of geranylgeraniol in a small subset of neurons is required for LTP and learning. These studies confirm the idea that at least some neurons not only are able to synthesize cholesterol, but its production is also essential for higher-order brain functions.

1.3.3. CYP46A1 and oxysterols in AD

As previously described, significant evidence points to a correlation between the onset of AD and imbalanced cholesterol homeostasis in the brain. Arising from the importance of CYP46A1 in the maintenance of this homeostasis in the CNS, an increasing number of studies also suggest that CYP46A1 could affect the pathophysiology of AD. The first evidence came from studies evaluating the potential use of 24OHC plasma levels as a biomarker for AD (Lutjohann *et al.* 2000). Elevated serum levels of 24OHC were detected in AD and vascular demented patients, compared to healthy controls, reflecting an increase in cholesterol turnover with neurodegeneration. Interestingly, in severely affected AD patients, a decrease in 24OHC levels was observed probably as a consequence of reduced levels of CYP46A1 due to neuronal loss (Papassotiropoulos *et al.* 2000). However, quantification of 24OHC in the CSF indicated that in AD patients an increase in 24OHC occurs, suggesting the possible use of this oxysterol as a biomarker for neurodegeneration (Papassotiropoulos *et al.* 2002). In the same study, a correlation between Apo ε4 allele and the levels of 24OHC was also described reinforcing the idea of altered cholesterol metabolism in AD associated with Apo E isoform. In AD brains reduced levels of 24OHC were detected concomitantly with increased 27OHC levels (Heverin *et al.* 2004), which is predominantly from extracerebral origin (Heverin *et al.* 2005). In the brain 27OHC is rapidly metabolized into 7a-hydroxy-3-oxo-4-cholestenoic acid, in a process mediated by the oxysterol 7α-hydroxylase (CYP7B1), and is excreted through the BBB (Meaney *et al.* 2007). Interestingly, CYP7B1 levels are decreased in AD brains (Yau *et al.* 2003).

A number of studies also suggest that 24OHC is able to modulate APP processing, and therefore have a protective role in AD (Fig. 1.3). It has been described that 24OHC inhibits Aβ secretion in primary cultures of cortical neurons in a higher rate than 27OHC (Brown *et al.* 2004). 24OHC also increases α-secretase activity as well as

the α/β-secretase activity ratio, whereas 27OHC antagonizes those effects (Famer *et al.* 2007). Moreover, in human neuroblastoma cells, 24OHC treatment increased APP processing through the non-amyloidogenic pathway, as well as ABCA1 and ABCG1 protein levels, which was associated with lower levels of Aβ (Prasanthi *et al.* 2009). On the other hand 27OHC appears to induce Aβ production by up-regulating APP and β-secretase protein levels. Initially, Ingemar Bjorkhem's group suggested that, as previously referred, 27OHC could be the missing link between hypercholesterolemia and AD, and the balance between 24OHC and 27OHC could modulate amyloidogenesis and neurodegeneration (Bjorkhem *et al.* 2009). Nevertheless, the same authors have recently shown that there is a 4-fold accumulation of 27OHC in different regions of the cortexes of patients carrying the Swedish APPswe 670/671 mutation, and concluded that accumulation of 27OHC is likely to be secondary to neurodegeneration, possibly a result of reduced activity of CYP7B1, the neuronal enzyme responsible for the metabolism of 27OHC (Shafaati *et al.* 2011a).

The first direct association of CYP46A1 enzyme in AD was described in a study from the same group, where a relative high concentration of CYP46A1 protein was detected in glial cells of AD brains, whereas its expression is restricted to neurons in healthy brains (Bogdanovic *et al.* 2001). This was later confirmed by others that also described the presence of CYP46A1 around neuritic plaques (Brown *et al.* 2004). In the last ten years, several groups have tried to establish an association between genetic variants of *CYP46A1* and the prevalence of AD. Despite the high number of studies developed so far, a clear conclusion is still missing. In some studies an association between intronic polymorphisms in *CYP46A1* and AD was described (Kolsch *et al.* 2002, Papassotiropoulos *et al.* 2003, Johansson *et al.* 2004, He *et al.* 2012), while in others it failed to be established (Desai *et al.* 2002, Chalmers *et al.* 2004, Tedde *et al.* 2006). Although the role of CYP46A1 in AD is not clear, in a promising study Hudry and co-workers demonstrated that *in vivo* overexpression of *CYP46A1* before and after the onset of amyloid plaques significantly reduces Aβ pathology in mouse models of AD (Hudry *et al.* 2010). Moreover, an improvement in spatial memory was also observed if the overexpression occurred before the onset of the plaques. HMGCR and SREBP2 were show to be slightly up-regulated as a compensatory mechanism of increased cholesterol catabolism and despite de two-fold increase in 24OHC; once more no changes were

observed in LXR-regulated genes. The reduced accumulation of Aβ is suggested to result from the decreased cholesterol content in lipid-rafts induced by *CYP46A1* overexpression, which modulates APP processing via the non-amyloidogenic pathway by decreasing γ-secretase activity (Simons *et al.* 1998, Wahrle *et al.* 2002). Nevertheless, the mechanism by which *CYP46A1* facilitates Aβ clearance remains to be fully determined. In a different study, ablation of ACAT1 in the triple transgenic mice model of AD lead to a reduction of more than 60% in the full-length APP and its proteolytic fragments, as well as an amelioration of cognitive deficits. Concomitantly, a significant increase in 24OHC levels and a decrease in HMGCR protein and cholesterol synthesis rate were also observed (Bryleva *et al.* 2010). The authors suggest that the increase in free cholesterol, as a consequence of ACAT1 depletion, induces CYP46A1 and 24OHC levels, which decreases APP levels and processing and HMGCR protein content, leading to amelioration of Aβ pathology. However, another study described that the loss of one or both cholesterol 24-hydroxylase alleles in a mice model of AD increased longevity, despite no changes were observed in amyloid plaque accumulation and APP processing (Halford & Russell 2009).

Clearly the reduction in cholesterol biosynthesis and catabolism does not confer any advantage in AD onset, but the protective effect of increased lifespan appears to be linked to cholesterol metabolism. Further studies are needed to determine the role of CYP46A1 in AD.

1.4. Transcriptional regulation of eukaryotic gene expression

In multicellular eukaryotic organisms, hundreds of different cell types carry out a range of specialized functions that depend upon the genes that are only switched on and off in that cell type, in each particular point in time. By complex regulatory mechanisms, the eukaryotic cell can control when and how often a particular DNA sequence is transcribed into RNA, how an RNA transcript is spliced or otherwise processed, can select which mRNAs are exported from the nucleus to the cytosol, which are degraded, and which are translated by ribosomes, and can introduce post-translational modifications in the protein into its functional form. Gene expression can therefore be regulated at each of these different steps (Voet *et al.* 1999).

Transcription is thus the first cellular event that allows the information present in the DNA to be translated into proteins or functional RNAs, and begins with the binding of RNA polymerases (RNA pol), the enzymes responsible for the synthesis of RNA molecules from a DNA template.

The DNA sequence of eukaryotic protein-coding genes that specifies where RNA pol II binds is referred as the core promoter and is usually located within the first 40 base pairs (bp) upstream or downstream of the transcription start site. The transcription process by RNA pol II involves three different stages: i) initiation, which is characterized by RNA pol II recognition and binding to the promoter of the gene; ii) elongation, in which the nascent RNA molecule is synthesized; and iii) termination that occurs when the RNA strand is complete and RNA pol II dissociates from DNA.

The initiation step is critical for gene transcription and is highly dependent on recruitment/presence of the transcription machinery and, as mentioned before, the recognition of the core promoter. The protein complex general transcription factor IID (TFIID), which is composed of TATA-binding protein (TBP) and 13 or 14 TBP-associated factors, recognizes and binds to core promoter elements (Baumann *et al.* 2010). In eukaryotic cells, these elements are diverse and the TATA box was the first to be described (Breathnach & Chambon 1981). Although the majority of promoters do not contain a canonical TATA box and several genes do not contain any of the known core promoter elements (i.e. initiator and CpG islands), evidence suggests that the subunits of TFIID recognize and bind virtually all the RNA pol II promoters (Goodrich & Tjian 2010). TFIID along with RNA pol II and other general transcription factors such as TFIIB, TFIIF, TFIIE and TFIIH, assemble the pre-initiation complex and transcription can be initiated. Apart from the core promoter, other regulatory elements are involved in the control of transcription. The proximal promoter (40 to 250 bp upstream of the transcription start site) contains *cis*-regulatory elements, which are DNA sequences recognized by specific transcription factors (*trans*-acting factors) that can further regulate transcription by acting as activators or repressors. Other protein-binding long-distance DNA motifs such as enhancers and silencers are also involved in the up-regulation or repression of transcription, respectively (Riethoven 2010). Therefore, the different regulatory elements present at a specific gene *locus*, and the different specific transcription factors that are expressed in a particular time frame, confer a combinatorial

control of transcription, which exponentially increases the potential number of unique expression patterns.

Despite the previously described importance of the recruitment of the transcriptional machinery and the binding of specific transcription factors, this is only possible if the particular genetic *loci* are accessible. By enabling or not the binding of transcription complexes and factors, the structure of chromatin is the primarily point of control in transcriptional regulation.

1.4.1. Regulation by the structure of chromatin

In eukaryotic cells DNA is packaged and condensed into chromatin in the nucleus. The nucleosome is the fundamental unit of chromatin, and is composed of 147 base pairs of DNA wrapped around an octamer of the four core histones (H) – H2A, H2B, H3 and H4 – along with one copy of the linker histone H1. The histone H1 is able to bind linker DNA and join nucleosomes together, regulating the packaging of DNA into higher-order structures. Therefore, chromatin is not uniform in structure and depends on the level of nucleosome packaging (Kornberg & Lorch 1999). For transcription to occur, chromatin must acquire a transient and more relaxed conformation through the displacement of nucleosomes to allow the binding of the transcription machinery. Due to its importance in the control of gene expression chromatin structure and activity is under tight regulation. Since the structure of chromatin is primarily dependent on nucleosome assemble and interaction, histone modifications have a direct effect on chromatin and represent a key regulatory mechanism. Both covalent modifications of histone proteins and the dynamic shuffling of histone variants can change nucleosome conformation and stability, by disrupting protein-DNA, protein-protein or nucleosome-nucleosome interactions. Furthermore, histone modifications can form binding surfaces for specific protein domains, and contribute to the recruitment of nonhistone protein complexes with enzymatic activity that induce alterations in the chromatin structure, the chromatin remodeling complexes (Kouzarides 2007, Smith & Shilatifard 2010). Moreover, DNA methylation, which consists in the addition of a methyl group directly to cytosine bases of CpG dinucleotides in the DNA template, and is usually associated with silent chromatin, can provide docking sites for proteins that can alter the chromatin state or affect the covalent modification of resident histones (Sasai & Defossez 2009, Bartke *et al.* 2010).

Recent evidence also suggests that long noncoding RNAs participate in *trans* in the regulation of chromatin state by recruiting chromatin remodeling complexes to specific genomic *loci* and inducing a repressive state of more compacted chromatin (Saxena & Carninci 2011). Thus, chromatin should be viewed as a dynamic polymer that ultimately determines which genes are expressed. In this section, we will focus on the effect of histone modifications on chromatin structure and on its functional consequences, with particular emphasis on histone acetylation.

Histones are small and globular proteins with an unstructured and flexible N-terminus, usually named "histone tail". A number of different post-translational modifications can occur in specific residues of the histone tail, although the globular and C-terminal domains can also be modified. The covalent modifications include acetylation, phosphorylation, methylation, monoubiquitination, sumoylation and ADP ribosylation. The most frequent are illustrated in Fig. 1.4.

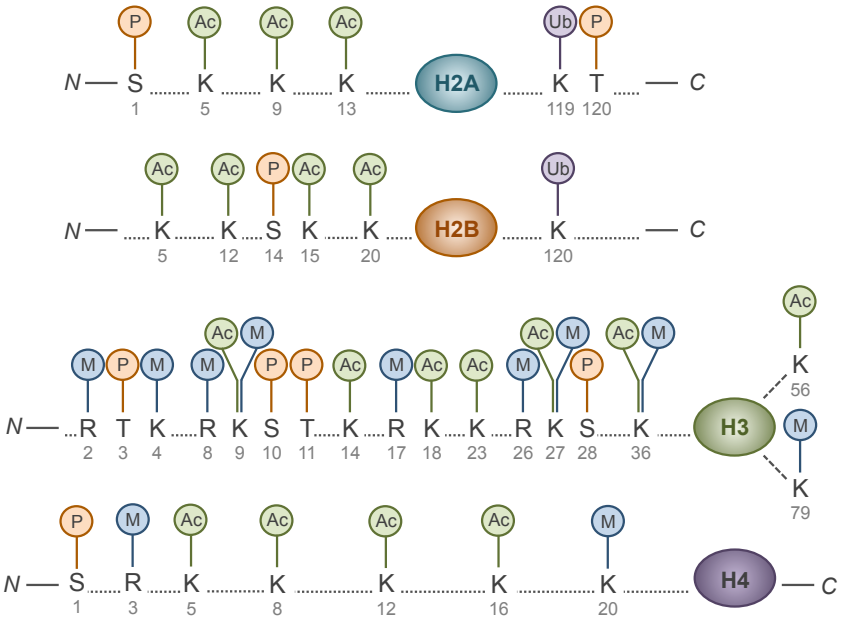

Figure 1.4. Post-translational modifications of core histones. Modifications include methylation (M), acetylation (Ac), phosphorylation (P) and ubiquitination (Ub). Although most of the modifications identified so far occur in the N-terminal tail, both the C-terminal tail and globular domain of some histones can also undergo post-translational modifications. K-lysine; R-arginine; S-serine; T-threonine. *Adapted from Bhaumik et al. (2007).*

Lysine (K) acetylation and methylation are the most well described modifications. While some modifications are associated with transcriptionally active chromatin (active marks), others correlate with transcriptionally inactive/silent regions (repressive marks). For instance, histone acetylation is usually associated with actively transcribed genes, while methylation can have different effects depending on the residue modified: methylation of histone H3 lysine 4 (H3K4) and H3 lysine 36 are active marks, while methylation of H3 lysine 9 (H3K9), H3 lysine 27 (H3K27), and H4 lysine 20 (H4K20) are repressive marks (Kouzarides 2007).

During the last ten years, a larger number of enzymes responsible for directing these covalent modifications have been identified, such as acetyl- and methyltransferases, kinases and ubiquitilases. Moreover, since the majority of histone modifications were found to be dynamic, in fact in some cases (i.e. acetylation, phosphorylation and methylation) changes can occur within minutes after a stimulus, the enzymes responsible for the removal of those specific modification have also been identified (i.e. deacetylases and demethylases) (Kouzarides 2007).

The impact of histone post-translational modifications on the functional state of chromatin can be regarded as a direct consequence of alterations in histone charge that affects histone-histone and histone-DNA interactions resulting in nucleosome disruption. Indeed, this is thought to be the case of acetylation, although it has been an experimental challenge to prove this *in vivo*. Nevertheless, it has been shown that *in vitro* acetylation of histone H4 lysine 16 (H4K16) negatively impacts the generation of compact fibers and the assembling in higher-order structures, as well as the interaction with nonhistone proteins (Shogren-Knaak *et al.* 2006). Phosphorylation has also been suggested to have the same effect although an *in vitro* demonstration is still lacking. The most well characterized mechanism of histone modifications function is the recruitment of nonhistone proteins, with enzymatic activity that, when recruited to histones through the recognition of specific binding domains (Fig. 1.5), are able to induce structural changes in chromatin and affect several biological processes (Taverna *et al.* 2007).

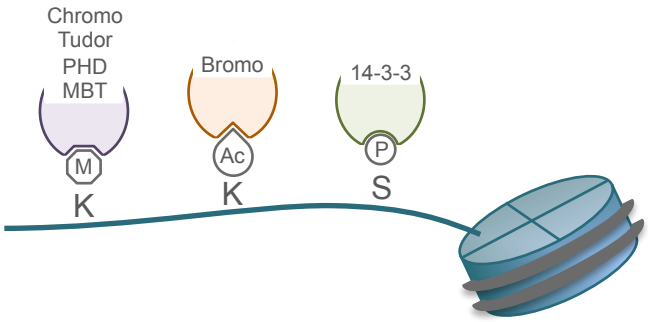

Figure 1.5. Specific binding domains involved in the recruitment of proteins to histones. The chromo-like domains of the Royal family (chromo, tudor and MTB) and nonrelated PHD domains recognize lysine (K) methylation (M), whereas lysine acetylation (Ac) is recognized by bromodomains and serine (S) phosphorylation (P) by a domain within 14-3-3 proteins. *Adapted from Kouzarides (2007).*

For instance, as a classic example we can refer to the heterochromatin protein 1 that binds methylated H3K9 through its chromodomain, and is associated with deacetylase and methyltransferase activity, in a process that maintains the heterochromatic silencing (Bannister *et al.* 2001, Lachner *et al.* 2001). Other example is the methylation of H3K27 repressor mark that recruits the chromodomain containing polycomb protein PC2, which is associated with ubiquitin ligase activity and chromatin compaction (Simon & Kingston 2009).

In summary, the functional consequences of histone modification range from the establishment of chromatin environments to the mediation of DNA-based processes such as transcription, repair and replication.

1.4.1.1. Histone acetylation

Acetylation is a post-translational modification that consists in the reversible addition of an acetyl group to the ε-amino group of a lysine residue within the core histone tail (Gershey *et al.* 1968). As previously mentioned, acetylation induces nucleosome disruption creating a more open and accessible chromatin conformation, that is usually associated with transcriptional activation. Furthermore, this specific modification is enriched in actively transcribed genes, particularly at specific sites in the promoter and 5' end of the coding regions (Clayton *et al.* 2006).

The acetylation level at a particular genomic region is highly dynamic and results from the net activity of histone acetyltransferases (HAT), and histone deacetylases

(HDAC). In transcriptionally active chromatin regions, the rates of histone acetylation and deacetylation are very high, while in silent regions the rate of reversible acetylation is slow (Clayton *et al.* 2006, Sun *et al.* 2001, Spencer & Davie 2001). Since the mid-1990s, when the first HATs and HDACs were identified, multiple enzymes with acetylase and deacetylase activity have been described. The study and identification of HATs in several species indicated that they are evolutionary conserved from yeast to humans and usually contain multiple subunits with transcriptional co-activator activity. Although when recruited to gene promoters, HATs increase the level of histone acetylation and enhance transcriptional activity, a number of non-histone proteins are also acetylated with important consequences in several cellular processes such as metabolism, DNA repair and replication, apoptosis and signaling (Lee & Workman 2007). From the several families of HATs already identified (Table 1.1) the three most important are the GNAT (Gcn5-related N-acetyltransferase, named after the founding member Gcn5*)*, p300/CBP (CREB-binding protein) and the MYST family (named for the founding members of this family: Morf, Ybf2, Sas2 and Tip60) (Selvi *et al.* 2010).

Table 1.1. HAT families.

HAT family	Histones modified
Type A HAT (nuclear HAT)	
1. GNAT family	H2B, H3, H4
2. p300/CBP family	H2A, H2B, H3
3. MYST family	H3, H4
4. Transcription factor related	H3, H4
5. Nuclear receptor associated	H3, H4
Type B HAT (cytoplasmic HAT)	H4

Adapted from (Selvi et al. 2010)

HDAC enzymes are also evolutionary conserved among many species. The mammalian genome encodes for 18 HDAC enzymes. Based on the sequence homology to the yeast HDAC, they are divided in 4 classes: class I HDACs includes HDAC1, 2, 3 and 8 (related to the yeast Rdp3 protein); class II HDACs include HDAC4, 5, 6, 7, 9 and 10 (related to the yeast HDA1 protein), and are further divided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10), according to their structural similarities; class III HDACs include SIRT1-7 and are referred as sirtuins due to the homology to the yeast

Sir2 protein; class IV HDAC is represented by HDAC11, the most recently identified HDAC isoform with a distinct structure. Classes I, II and IV HDACs are zinc-dependent enzymes (Fig. 1.6) whereas class III HDACs are dependent on nicotinamide adenine dinucleotide (NAD^+) (Yang & Seto 2007).

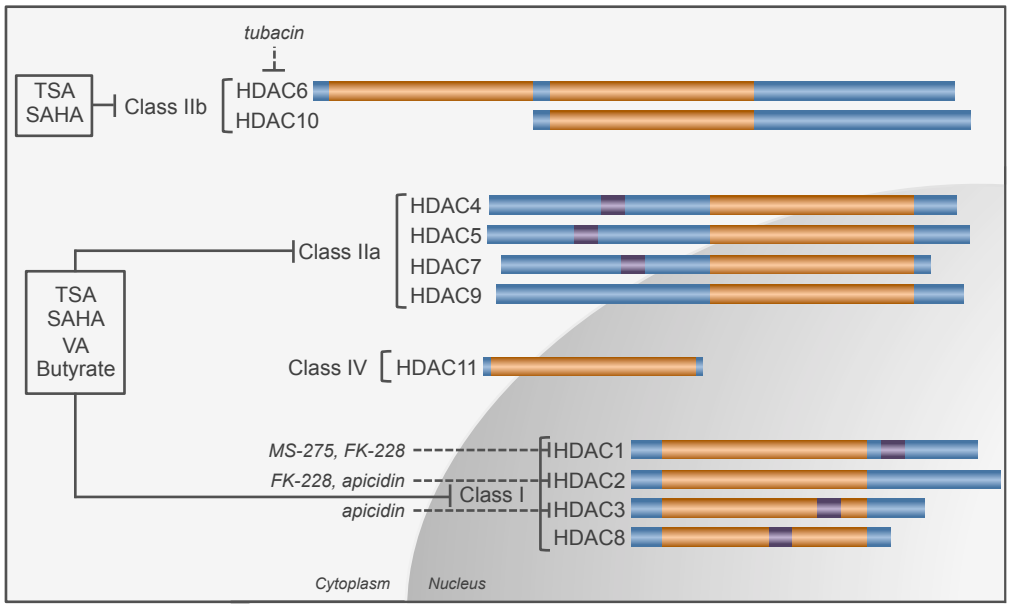

Figure 1.6. Schematic representation of zinc-dependent HDACs and its inhibitors. The subcellular localization of each HDAC is presented along with the pan-inhibitors and the more specific ones. The deacetylase catalytic domain (orange) and nuclear localization signal (purple) are depicted. TSA-trichostatin A; SAHA-suberoylanilide hydroxamic acid; VA-valproic acid. *Adapted from Chuang et al. (2009) and Karagiannis & Ververis (2012).*

HDACs, like HATs, also target non-histone proteins and both classes of enzymes do not bind directly to DNA. Instead, HDAC and HAT function is exerted through co-repressor or co-activator complexes, respectively, that are recruited to the promoter region of genes by transcription factors (section 1.4.2). The activity of these enzymes, which can be modulated by signaling mechanisms comprise another degree of transcriptional regulation. Recruitment of CBP to the transcriptional complex has been described to depend upon direct phosphorylation (Zanger *et al.* 2001), and both CBP and p300 are phosphorylated by extracellular signal-regulated kinase (ERK) 1/2 members of the mitogen activated protein kinase (MAPK) family of signaling pathways upon nerve growth factor treatment in neuronal cells (Liu *et al.* 1999) and in cardiac cells after phenylephrine treatment (Gusterson *et al.* 2002). Overall, HAT phosphorylation promotes

the recruitment to transcriptional complexes and enhances their acetyltranferase activity with direct consequences on transcriptional activation (Saha & Pahan 2006). Furthermore, both HDAC1 and HDAC2 are phosphorylated by casein kinase 2, which promotes enzymatic activity and complex formation (Pflum *et al.* 2001, Tsai & Seto 2002).

The function of HDACs in cellular processes has been greatly elucidated by the use of chemical inhibitors of their enzymatic activity. Two major classes of HDAC inhibitors (HDACi) presently exist: the hydroxamic acids, such as trichostatin A (TSA) and SAHA (suberoylanilide hydroxamic acid), which are non-selective HDACi that inhibit most zinc-dependent HDACs, and the carboxylic acids (e.g. sodium butyrate and valproic acid) that inhibit both class I and IIa HDACs, but are weaker inhibitors than TSA and SAHA. Furthermore, compounds that inhibit HDACs more specifically also exist. The class of cyclic peptides that include apicidin and FK-228 primarily inhibit HDAC2/HDAC3 and HDAC1/HDAC2, respectively, and the MS-275 benzamide preferentially targets HDAC1 (Xu *et al.* 2007). Because HDACi promotes histone acetylation, treatment with these compounds is often associated with increased gene expression. However, studies using DNA arrays demonstrated that only a small percentage of genes have altered expression after HDACi treatment (7% to 10%), with approximately the same number of genes being up- and down-regulated (Mitsiades *et al.* 2004, Peart *et al.* 2005, Glaser *et al.* 2003, LaBonte *et al.* 2009).

1.4.1.2. HDACs and HDAC inhibitors in neurodegenerative conditions

In recent years much effort has been made to understand if deregulation of epigenetic mechanisms, namely an imbalance in acetylation homeostasis, may contribute to neurodegenerative conditions (Saha & Pahan 2006). In fact, while reduced histone acetylation levels triggered by the loss of CBP HAT are associated with neurodegeneration (Rouaux *et al.* 2003), the beneficial effects of HDACi treatment in several neurodegenerative disorders strongly supports the idea of neuronal acetylation homeostasis loss during neurodegeneration. For instance, HDACi have been shown to have neuroprotective, neurotrophic and anti-inflammatory properties, while improving neurological performance and learning/memory in several disease animal models of HD (Ferrante *et al.* 2003, Hockly *et al.* 2003, Gardian *et al.* 2005), spinal muscular atrophy

(Chang *et al.* 2001, Avila *et al.* 2007), amyotrophic lateral sclerosis (Corcoran *et al.* 2004, Ryu *et al.* 2005, Petri *et al.* 2006), experimental autoimmune encephalomyelitis (Camelo *et al.* 2005), and AD (Qing *et al.* 2008, Francis *et al.* 2009, Ricobaraza *et al.* 2012). The beneficial effects of HDACi treatment are very diverse. In HD, which is characterized by altered gene expression (Sugars & Rubinsztein 2003), transcriptional balance is restored after HDACi treatment. Mutant huntingtin interacts with CBP and reduces its acetyltransferase activity, inducing a global histone deacetylation (Steffan *et al.* 2001) that is reversed by overexpressing CBP or treatment with HDACi (McCampbell *et al.* 2001). Furthermore, treatment of R6/2 model of HD with HDACi corrected histone hypoacetylation and the transcriptional abnormalities observed (Sadri-Vakili *et al.* 2007, Thomas *et al.* 2008).

Moreover, cognitive decline, a feature of most neurodegenerative disorders that results from synaptic plasticity and long-term memory impairment, also appears to be modulated by HDACi. Memory formation is a complex process that requires gene expression, through *de novo* RNA and protein synthesis (Alberini 2009). Therefore, a change in histone acetylation and consequently modulation of transcription patterns appears to be an integral part of synaptic plasticity, and CBP HAT activity has a critical function in long-term memory formation (Guan *et al.* 2002), since its ablation results in both reduced histone acetylation and memory impairment. Moreover, CBP is necessary for specific hippocampus-dependent long-term memory (Martin & Sun 2004, Korzus *et al.* 2004).

On the other hand, HDACs have been shown to have the opposite effect, acting as negative regulators of long-term memory processes. Indeed, despite the requirement of both HDAC1 and HDAC2 for multiple aspects of neuronal development (Montgomery *et al.* 2009), overexpression of HDAC2 but not HDAC1 resulted in decreased dendritic spine density, synapse number, synaptic plasticity and memory formation, while *Hdac2* deficiency resulted in increased synapse number and memory facilitation. Furthermore, HDACi treatment was able to ameliorate the effects of HDAC2 overexpression, suggesting that although HDAC2 functions as a memory suppressor, specific targeting of this HDAC could represent a way to treat cognitive disorders (Guan *et al.* 2009). Moreover, treatment with non-selective HDACi was shown to induce sprouting of dendrites, increase number of synapses, and reinstate learning behaviour and access to long-term memories (Fischer *et al.* 2007). HDACi also modulates long-term memory for

novel object recognition (Stefanko *et al.* 2009). Interestingly, in a very recent study the cognitive decline observed in a mouse model of AD was attributed to an epigenetic blockade of gene transcription due to increased expression of HDAC2, which was also observed in post-mortem brain samples of AD patients. Most importantly, reverting the increased HDAC2 expression unlocked gene repression and restored synaptic plasticity, suggesting that selective inhibitors of HDAC2 may partially restore cognitive capacities following neurodegeneration (Graff *et al.* 2012). Also, in a mouse model of HD the cognitive deficits detected were associated with reduced hippocampal expression of CBP and diminished levels of histone H3 acetylation, with consequences in the expression of target genes involved in memory formation. Once more, HDACi treatment rescued recognition memory deficits, as well as CBP-mediated gene expression (Giralt *et al.* 2012). In addition, HDAC3 have also been established to negatively regulate long-term memory formation. Deletion of *Hdac3* in rat hippocampus resulted in increased histone acetylation and enhanced long-term memory associated with increased expression of genes involved in memory formation (*Nr4e2* and *c-fos*) (McQuown *et al.* 2011).

Besides the established beneficial effects of HDACi treatment in neurodegenerative conditions, unwanted consequences may occur, from cellular toxicity to distinct effects depending on neuronal cell type (Dietz & Casaccia 2010). Although the idea of using HDACi for neurodegenerative disorders treatment appears to be very promising, the current opinion is that specific HDAC isoforms involved in the diverse neurological disorders must be identified, as well as the brain regions affected by the process of neurodegeneration, but more importantly selective inhibitors must be developed in order to prevent undesirable side effects.

1.4.2. Regulation by transcription factors

As previously mentioned, assemble of the pre-initiation complex at the core promoter, which contains the basal transcriptional machinery composed of RNA pol II and general transcription factors, is essential for transcription initiation. Nevertheless, several sequence-specific DNA-binding transcription factors that recognize and bind distinct regulatory regions are crucial for the modulation and specificity of the transcription program (Kadonaga 2004). These transcription factors are typically modular and share several similarities: the presence of a DNA binding domain (DBD) that

recognizes a specific nucleotide sequence (responsive element); one or more activation/repressor domains, which interact with co-regulators or general transcription factors; a multimerisation and a regulatory domain (Kadonaga 2004).

The current view is that the binding of these transcription factors to specific DNA sequences in response to cellular signals can modulate transcription by the recruitment of general transcription factors or transcriptional co-activators/repressors. Those players serve as a bridge between sequence-specific factors and the basal/general transcription machinery, which consequently modulate the pre-initiation complex assemble and RNA pol II activity. Furthermore, sequence specific transcription factors are responsible for the recruitment of chromatin remodeling complexes (i.e. ATP-dependent enzymes that mobilize nucleosomes) as well as co-activator/repressor complexes with histone modifying enzymes, directly affecting chromatin structure (Kadonaga 2004, Venters & Pugh 2009). Transcription factors can recruit several multiprotein complexes with histone deacetylase activity. Indeed, both HDAC1 and HDAC2, the best-characterized HDACs, are usually associated with three complexes, the Sin3, NuRD/NRD/Mi2 and CoREST, while HDAC3 forms the N-CoR1 repressor complex (Sengupta & Seto 2004). Particularly, CoREST is involved in the silencing of neuronal genes in non-neuronal cells and acts as a co-repressor of REST/NRSF (neuron-restrictive silencer factor) transcription factor (Andres *et al.* 1999). Protein-protein interactions between HDACs and the proteins that form these large complexes are essential for the regulation of HDAC enzymatic activity, which present better deacetylase activity in the complexes (Sengupta & Seto 2004).

Modulation of transcription factors activity by post-translational modifications also represents a mechanism of gene transcription regulation; particularly, phosphorylation by the MAPK signaling cascade has been widely described. This specific modification can regulate transcription factors intracellular localization, protein levels, DNA-binding activity and interaction with other regulatory proteins (Whitmarsh 2007).

1.4.2.1. The family of Sp transcription factors

Specificity protein (Sp) 1 was one of the first mammalian sequence-specific transcription factors to be identified and characterized (Kadonaga *et al.* 1987). This protein was described as having the ability to bind to GC-boxes (GGGGCGGGG) in the promoter of genes leading to transcriptional activation (Dynan & Tjian 1983, Jones *et al.* 1985), and for several years was regarded as a versatile general transcription factor. The ubiquitously expressed Sp1 was found to present three Cys2His2 type zinc-fingers as DBD motifs, along with two glutamine-rich activation domains, and to interact with several proteins from general transcription factors to cell cycle regulators (Suske 1999). Moreover, the discovery of proteins highly homologous to Sp1 in mammalian cells established that Sp1 was just one member of the family. The specificity protein/Krüppel-like factor (Sp/KLF) family of transcription factors comprises the Sp- and the KLF-family, whose members share a highly conserved DBD of three adjacent Cys2His2 type zinc-fingers and are able to bind both GC- and GT-boxes (GGTGTGGGG). A characteristic of the Sp members is the presence of a Buttonhead domain, just N-terminal to the DBD (Suske *et al.* 2005).

So far nine members of the Sp-family were identified (Sp1-9), however the members of the Sp1-4 subgroup not only share a similar modular structure but also contain N-terminal glutamine-rich transactivation domains, unlike Sp5-9. Nevertheless, Sp1, Sp3 and Sp4 apart from being the most-well characterized members are more closely related, with two transactivation domains and the ability to bind both GC- and GT-boxes, whereas Sp2 with only one transactivation domain is unable to bind GC-boxes (Wierstra 2008). Furthermore, while Sp1 and Sp3 are ubiquitously expressed, Sp4 is specifically expressed in the brain (Hagen *et al.* 1992). Sp3 and Sp1 also have an inhibitory domain and can both act as positive or negative regulators of gene expression depending on the cellular and promoter context, although Sp1 is regarded as a stronger activator than Sp3 (Suske 1999). Particularly the Sp3 shorter isoforms, that lack one of the transactivation domains, have been described as repressors (Kennett *et al.* 1997, Kennett *et al.* 2002). Although literature of Sp4 is scarce, it has been described so far as an activator (Suske 1999). Functionally, Sp1 but not Sp3 and Sp4 has the capacity to form multimers and synergistically activate transcription through adjacent binding sites, and in those conditions Sp3 can repress Sp1-mediated activation by competing for the same

responsive elements (Yu *et al.* 2003). Moreover, Sp3 can also repress Sp4-mediated transcriptional activity, which is strongly enhanced ("superactivated") in the presence of Sp1, suggesting that Sp1 and Sp4 can directly interact and Sp4 can function as a target for the Sp1 activation domain (Hagen *et al.* 1995). Two important mechanisms regulate Sp factors transcriptional activity, which are the interaction/cooperation with other proteins and post-translational modifications that directly affect their activity. We will focus on Sp1 and Sp3 because they are the most extensively studied.

Both Sp1 and Sp3 directly or indirectly interact with general transcription factors and with sequence-specific transcription factors, which enhance the initiation complex assemble to initialize transcription. Particularly Sp1 was found to interact with TBP, estrogen receptor and p53 (Li *et al.* 2004). Moreover, and as previously mentioned, transcription factors have the ability to modulate transcription through the recruitment of complexes that alter chromatin structure and that is the case of Sp proteins. Sp1 can recruit the ATP-dependent chromatin remodeling complex SWI/SFN to promote chromatin accessibility to the transcription machinery (Lu *et al.* 2003), and both Sp1 and Sp3 can recruit repressor complexes such as Sin3A HDAC1/HDAC2 (Zhang & Dufau 2003, Clem & Clark 2006), or co-activators such as the CBP and p300 HATs to enhance transcription (Ammanamanchi *et al.* 2003, Jang & Steinert 2002).

Several post-transcriptional modifications that include phosphorylation, acetylation, glycosylation and sumoylation have been described to affect Sp1/Sp3 activity, and hence gene transcription. Phosphorylation of Sp1, which mainly occurs in the DBD, and Sp3, appears to facilitate transcriptional activation possibly by facilitating the interaction with DNA (Waby *et al.* 2008, Pages 2007). Furthermore, while acetylation has been described to both activate (Ammanamanchi *et al.* 2003) and repress (Braun *et al.* 2001) Sp3 transcriptional activity, it seems that in Sp1 it usually increases transcriptional activity. Sp1 can be acetylated by p300, although the exact role of this modification remains unclear (Suzuki *et al.* 2000). At last sumoylation, which typically have a negative impact on transcription factors activity (Verger *et al.* 2003), have also been described to repress Sp1- (Spengler & Brattain 2006, Wang *et al.* 2008b) and Sp3-mediated transcription (Ross *et al.* 2002, Stielow *et al.* 2008).

The mechanisms involved in the modulation of transcription by the ubiquitously expressed Sp family of transcription factors are very diverse, from the recruitment of general transcription factors to chromatin remodeling complexes, reflecting the wide

range of processes regulated by these proteins and the variability of transcriptional outcomes. Nevertheless, it urges to increase the knowledge of how these transcription factors interact with chromatin remodeling complexes in the context of neuronal chromatin states, in different physiological and pathological conditions, which may have important consequences in neuronal fate.

OBJECTIVES

The work presented in this thesis is focused on the characterization of the regulatory pathways that control *CYP46A1* expression, a neuronal-specific cytochrome P450, particularly the participation of an epigenetic program involving histone acetylation. The main goal was therefore to determine the effect of HDAC inhibition in the transcriptional regulation of *CYP46A1* gene and to characterize the molecular mechanism underlying such effect. The critical importance of the CYP46A1 enzyme in brain cholesterol homeostasis, as the major pathway for brain cholesterol turnover, lead us to question if HDAC inhibition could also affect cellular cholesterol content and the expression levels of other key genes involved in cholesterol metabolism.

The experimental approaches used involve a wide range of cell and molecular biology techniques and were designed to answer the following specific questions:

1. Is *CYP46A1* gene expression regulated by epigenetic mechanisms such as histone acetylation?
2. Which molecular mechanisms underlie *CYP46A1* response to HDACi?
3. Which signaling transduction pathways participate in the response of *CYP46A1* gene to HDAC inhibition?
4. Are other genes involved in cholesterol metabolism modulated by HDAC inhibition?
5. Does HDAC inhibition affect cellular cholesterol content in neuronal cells?

Taken together, the results present herein further elucidate the molecular mechanisms underlying the effect of HDAC inhibition on the regulation of *CYP46A1* expression and consequently on brain cholesterol homeostasis. Moreover, by characterizing the effect of HDACi in the cholesterol metabolism of neuronal cells, these studies also provide a significant contribution regarding the complex regulation of brain cholesterol metabolism and define HDACi as a valuable therapeutic approach in neurodegenerative disorders with altered cholesterol metabolism.

CHAPTER 2

SP PROTEINS PLAY A KEY ROLE IN HISTONE DEACETYLASE INHIBITOR-MEDIATED DEREPRESSION OF *CYP46A1* GENE TRANSCRIPTION

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2.1. Abstract

We investigated whether the *CYP46A1* gene, a neuronal-specific cytochrome P450, responsible for the majority of brain cholesterol turnover, is subject to transcriptional modulation through modifications in histone acetylation. We demonstrated that inhibition of histone deacetylase activity by trichostatin A (TSA), valproic acid and sodium butyrate caused a potent induction of both *CYP46A1* promoter activity and endogenous expression. Silencing of Sp transcription factors through specific siRNAs, or impairing Sp binding to the proximal promoter, by site-directed mutagenesis, led to a significant decrease in TSA-mediated induction of *CYP46A1* expression/promoter activity. Electrophoretic mobility shift assay, DNA affinity precipitation assays and chromatin immunoprecipitation assays were used to determine the multiprotein complex recruited to the *CYP46A1* promoter, upon TSA treatment. Our data showed that a decrease in Sp3 binding at particular responsive elements, can shift the Sp1/Sp3/Sp4 ratio, and favor the detachment of histone deacetylase 1 (HDAC1) and HDAC2 and the recruitment of p300/CBP. Moreover, we observed a dynamic change in the chromatin structure upon TSA treatment, characterized by an increase in the local recruitment of euchromatic markers and RNA polymerase II. Our results show the critical participation of an epigenetic program in the control of *CYP46A1* gene transcription, and suggest that brain cholesterol catabolism may be affected upon treatment with HDAC inhibitors.

2.2. Introduction

The importance of understanding cholesterol metabolism in the central nervous system is underscored by the accumulating evidence that an association exists between altered cholesterol homeostasis and the development of neurodegenerative disorders such as Alzheimer's disease. The CYP46A1 enzyme has been identified as a neuronal-specific cytochrome P450, responsible for the conversion of cholesterol into 24(S)-hydroxycholesterol (24OHC) (Lund *et al.* 1999). The flux of this oxysterol across the blood-brain barrier is believed to be the main mechanism by which the brain facilitates the removal of cholesterol (Bjorkhem *et al.* 1998, Xie *et al.* 2003). *Cyp46a1* null mice exhibit severe deficiencies in spatial, associative and motor learning, and hippocampal long-term potentiation (Kotti *et al.* 2006). Moreover, *in vitro* evidence suggests that 24OHC can affect the processing of the amyloid precursor protein. Indeed, overexpression of CYP46A1, or treatment with 24OHC increases the α-secretase activity as well as the α/β-secretase activity ratio (Famer *et al.* 2007, Prasanthi *et al.* 2009). Importantly, Hudry and co-workers have recently demonstrated that *in vivo* overexpression of CYP46A1 before and after the onset of amyloid plaques significantly reduces amyloid β pathology in mouse models of Alzheimer's disease (Hudry *et al.* 2010).

Characterization of the human *CYP46A1* gene promoter has provided insights into the regulatory mechanisms of *CYP46A1* gene transcription. The promoter has no canonical TATA or CAAT boxes and is very GC-rich, a feature often found in genes considered to have a largely housekeeping function (Ohyama *et al.* 2006, Milagre *et al.* 2008). There seems to be no substrate-dependent transcriptional regulation and treatment of neuroblastoma cells with a broad spectrum of endogenous and exogenous compounds did not result in any significant change in *CYP46A1* promoter activity (Ohyama *et al.* 2006). Our previous studies have mapped a region spanning from nucleotides -236/-64 that is indispensable for basal expression of this gene, and have suggested that the cell-type specific expression of Sp transcription factors - substitution of Sp1 by Sp4 in neurons - is responsible for the basal expression of the *CYP46A1* gene (Milagre *et al.* 2008).

On the other hand, the fact that *CYP46A1* promoter reporter constructs present high luciferase activity in cell lines where CYP46A1 mRNA could not be detected, and

that these recombinants were strongly transactivated *in vitro* not only by the brain-specific Sp4, but also by Sp1 and Sp3, which are ubiquitously expressed transcription factors, suggested that *CYP46A1* gene silencing might be caused by epigenetic modifications such as DNA methylation and/or histone modifications. Although transcriptional regulation is highly complex and dynamic, increased histone acetylation can cause remodeling of chromatin from a tightly- to a loosely-packed configuration, which subsequently leads to transcriptional activation (for review see Kouzarides 2007). Conversely, a decrease in histone acetylation may cause chromatin structure to condense and results in transcriptional silencing. Therefore, up-regulation of transcription can be achieved in cells either by stimulation of histone acetyltransferases (HAT) or by inhibition of histone deacetylases (HDAC) activities, and the opposite is true for transcriptional down-regulation. Although histone acetylation is related to gene activation, global inhibition of HDACs does not induce widespread transcription (Van Lint *et al.* 1996, Eickhoff *et al.* 2000, Della Ragione *et al.* 2001). For instance, treatment of human lymphoid cell line with the HDAC inhibitor trichostatin A (TSA), revealed a change of expression in only 8 out of 340 genes (Van Lint *et al.* 1996), while other reports shown 5% to 10% change in total number of genes that were up- or down-regulated by HDAC inhibitors (Eickhoff *et al.* 2000, Daly & Shirazi-Beechey 2006).

In this study, we have investigated the effect of HDAC inhibitors on *CYP46A1* gene expression and characterized the molecular mechanisms underlying TSA-mediated derepression of the *CYP46A1* gene.

2.3. Methods

2.3.1. Antibodies

The antibodies used in chromatin immunoprecipitation (ChIP), electrophoretic mobility shift assay (EMSA) and Western blot (WB) are listed in Table 2.1.

Table 2.1. List of the antibodies used in ChIP, EMSA and WB.

Protein	Clone/Reference	Assay
Sp1	39058[1]	ChIP
Sp1	PEP-2[2]	EMSA, WB
Sp3	D-20[2]	ChIP, EMSA, WB
Sp4	V-40[2]	ChIP, EMSA, WB
HDAC1	05-100[3]	ChIP, WB
HDAC2	ab7029[4]	ChIP
HDAC2	05-814[3]	WB
CBP	ab2832[4]	ChIP
p300	05-257[3]	ChIP
RNA polymerase II	05-623[3]	ChIP
diacetylated histone 3	06-599[3]	ChIP
tetraacetylated histone 4	06-598[3]	ChIP
trimethylated histone 3 lysine 4	ab8580[4]	ChIP
dimethylated histone 3 lysine 4	ab7766[4]	ChIP
GAPDH*	6C5[2]	WB

1. Active Motif Inc. (Rixensart, Belgium)
2. Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
3. Upstate Biotechnology (Lake Placid, NY, USA)
4. Abcam Inc. (Cambridge, MA, USA)
** GAPDH - Glyceraldehyde-3-phosphate dehydrogenase*

2.3.2. Cell culture, reporter gene constructs and transactivation assays

SH-SY5Y (human neuroblastoma), HeLa (cervix adenocarcinoma) and *Drosophila* melanogaster Schneider (SL2) cell lines were maintained and transiently transfected as previously described (Milagre *et al.* 2008). The different recombinant *wild type* and mutated plasmids, derived from the 5' flanking region of the human CYP46A1 gene, and used in this work, have also been described previously (Milagre *et al.* 2008).

2.3.3. *CYP46A1* expression analysis

Total RNA from treated or untreated cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. 1.5 µg of RNA was reversed transcribed using SuperScript II reverse-transcriptase kit with random hexamer primers (Invitrogen), and first strand DNA from 150 ng of RNA was used as a template in quantitative real-time PCR (qPCR) with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). The cycling program was set as follows: denature at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and

60ºC for 1 min. The assay IDs used were: Hs00198510_m1 for CYP46A1 and Hs99999_m1 for human β-actin (Applied Biosystems). Results presented are from three individual experiments and each sample was assayed in triplicate. CYP46A1 mRNA levels were normalized to the level of β-actin and expressed as pg of CYP46A1 mRNA per ng of β-Actin mRNA (HeLa cells), or as fold change from controls, using the ΔΔCt method (SH-SY5Y cells).

2.3.4. Histone isolation

Cells were washed with phosphate-buffered saline (PBS) and harvested by centrifugation. The pellet was resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl pH=8.1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and protease inhibitor cocktail) and incubated for 30 min at 4ºC. The nuclei were isolated by centrifugation, resuspended in 0.4 N H_2SO_4 and incubated for 30 min at 4ºC. After nuclear debris removal, histones were precipitated with trichloroacetic acid at the final concentration of 33%, and collected by centrifugation. The pellet was washed twice with ice-cold acetone, dissolved in water and stored at -80ºC.

2.3.5. Western blot analysis

Nuclear extracts were prepared as described by Schreiber and co-workers (Schreiber *et al.* 1989). Nuclear proteins were subject to 10% SDS-PAGE gels and electroblotted onto Immobilon P (Millipore, Bedford, MA, USA). After visualization of the transferred proteins by amido black staining, the membranes were incubated with specific antibodies. Results were quantified using the Quantity One version densitometry analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.3.6. Small interfering RNA analysis

Pools of siRNAs designed to knock down the endogenous expression of Sp1, Sp3, Sp4 and siRNA for the negative control (scram) were obtained from Ambion (Ambion, Inc., Austin, TX, USA). siRNA transfections were performed with X-tremeGENE siRNA Transfection Reagent (Roche) according to the procedures

recommended by the manufacturer. Briefly, the X-tremeGENE reagent was diluted in Opti-MEM I medium (Invitrogen) and incubated at room temperature for 4 min. This was then mixed with a pool of the different siRNAs (100 nM), followed by incubation at room temperature for 30 min. The complexes formed were added to 2 x 10^6 cells in suspension, and plated in 60 mm culture dishes. At 24 h post-transfection, cells were replaced with fresh medium and grown for an additional 48 h before harvest. The cells from each dish were harvested and the cell pellet was used to prepare nuclear extracts and total RNA, as already described.

2.3.7. Electrophoretic mobility shift assay

EMSA was performed as previously prescribed (Milagre *et al.* 2008). Briefly, 2-5 μg nuclear extracts were incubated with γ^{32}P-labeled oligonucleotide probe harboring sites SP-RE-B or SP-RE-D. For supershift assay, 1μl of each specific antibody, or both were pre-incubated with the nuclear extracts for 30 min before the addition of probe. Protein-DNA complexes were resolved on 5% native polyacrylamide gels and visualized by autoradiography.

2.3.8. DNA affinity precipitation assay

A fragment of the human *CYP46A1* proximal promoter corresponding to the sequence -152 to -64 (+1 refers to the A of the initiation methionine), that encompasses sites Sp-RE-A and Sp-RE-B, was amplified by PCR using 5' biotin labeled primers, 5'-CGGCGGGGGCGGAGCCGAGC-3' and 5'-AAACCCGGGGGAGGGCCCGG-3', and *Pfx* polymerase (Invitrogen). The PCR amplified fragments were separated by agarose electrophoresis, purified on QIAquick columns (Gel extraction kit, Qiagen, Valencia, CA, USA) and used as a probe. DAPA was carried out by immobilizing 500 ng of the biotinylated probe per sample onto Dynabeads® M-280 Strepatvidin beads, as recommended by the manufacturer. Nuclear extracts from vehicle or TSA-treated cells at the indicated time points (50 μg/sample) were then incubated overnight, with gentle rotation, at 4ºC with the beads, in 400 μl of binding buffer (60 mM KCl, 12 mM Hepes pH= 7.9, 4 mM Tris-HCl pH=7.4, 5% (v/v) glycerol, 1 mM EDTA, 1 mM DTT plus protease inhibitor cocktail). The precipitated DNA-protein complexes were then washed

five times with binding buffer, resolved on 10% SDS-PAGE, and detected by western blot using specific antibodies.

2.3.9. Chromatin immunoprecipitation

ChIP assays were performed as described previously (Schnekenburger *et al.* 2007). The recovered DNA was analyzed by qPCR with SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems). The qPCRs were performed using primers that covered different regions of the promoter: 5' GCGGACCTGAGTCTGAAGAG 3' (forward) and 5' AATCACAACTCCGCTTCTGG 3' (reverse) for the proximal promoter (+1 region), 5' GGGAAGCCCTGGTCATTATT 3' (forward) and 5' GTTGGAGTTGGAGGGATGAA 3' (reverse) for the distal promoter (-1kb region), and 5' GTGTTGCCAGGTCTTCCAAT 3' (forward) and 5' GTGTCCGTGGTGAATGAGTG 3' (reverse) for a region located 11kb upstream of the initiation site.

2.3.10. Statistical analysis

Statistical analysis was performed using the Student's *t*-test and the ANOVA one-way test with the Tukey HSD *post-hoc* test or the Tukey HSD for unequal N (Spjotvoll/Stoline test). All analysis were performed using the STATISTICA (data analysis software system), version 7.1 StatSoft, Inc. (2006).

2.4. Results

2.4.1. Histone deacetylase inhibitors activate the transcription of human *CYP46A1* gene

In this study, we aimed to investigate the molecular mechanisms by which HDAC inhibitors induce the human *CYP46A1* gene. In order to investigate whether transcription of the *CYP46A1* gene was subject to modulation through chromatin modifications related to histone acetylation and deacetylation, transient transfection

studies using reporter gene analysis of the human *CYP46A1* promoter activity were performed in cells that do not express CYP46A1 mRNA. HeLa cells were transfected with *CYP46A1* reporter plasmids and treated or not with TSA, one of the most potent of the HDAC inhibitors yet identified.

The *CYP46A1* promoter activity was significantly increased by TSA (Fig. 2.1). Interestingly, the smaller promoter constructs, 0.12pGL2 (-236 to -64) and 0.09pGL2 (-152 to -64), already displayed 15- and 10-fold activation by TSA, respectively (Fig. 2.1A).

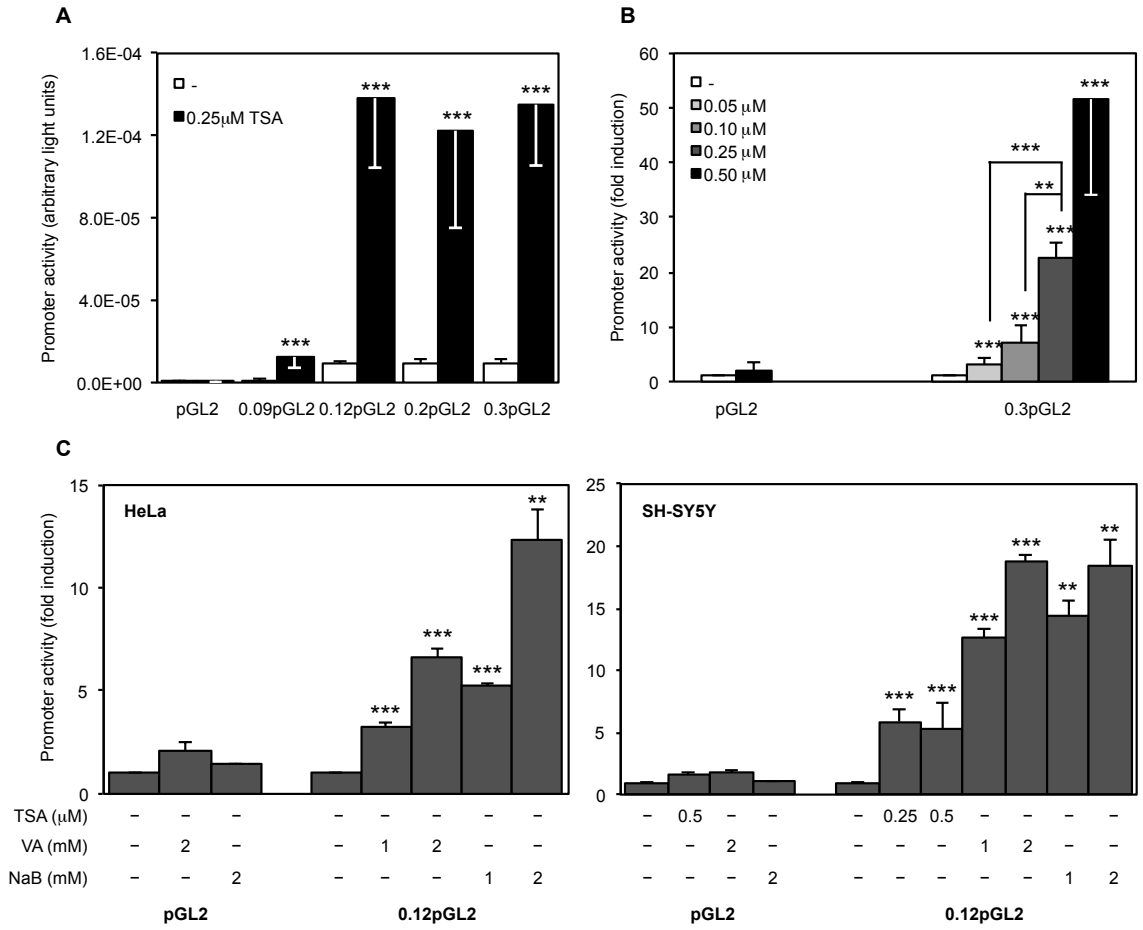

Figure 2.1. Histone deacetylase inhibitors activate the *CYP46A1* promoter. The *wild type* CYP46A1 promoter/reporter gene constructs (CYP46pGL2) and the promoter less vector plasmid (pGL2), were transfected into HeLa (A-C) or SH-SY5Y cells (C). Twenty-four hours after transfection, cells were incubated with the indicated concentrations of TSA (A-C), NaB (C), or VA (C) for 24 h. Relative promoter activity is indicated as arbitrary light units (A) or as fold induction over the promoter activity in the absence of treatment (B and C). Normalized luciferase activities were expressed as mean values ± SEM of duplicates for a minimum of three independent experiments (** $p < 0.01$; *** $p < 0.001$).

The *post-hoc* comparisons revealed that significant differences in the fold induction levels were found between pGL2 and the promoter constructs (ANOVA one-way test: $F = 13.61$, $df = 4$, $p < 0.001$; Tukey HSD for unequal N, $p < 0.01$). Further analysis showed a dose-dependent activation of the *CYP46A1* promoter activity by TSA (Fig. 2.1B). To further investigate whether induction of *CYP46A1* by TSA was mediated through inhibition of HDAC activity, we examined the effect of other HDAC inhibitors on the *CYP46A1* promoter activation, by comparing the effect of TSA with that of the antiepileptic drug valproic acid (VA) and of sodium butyrate (NaB). The 0.12pGL2 construct was transfected not only in HeLa cells, but also in cells where the CYP46A1 mRNA could be detected although at very low levels, the SH-SY5Y neuroblastoma cells. Our results show a significant activation of the *CYP46A1* promoter elicited by all the tested HDAC inhibitors (Fig. 2.1C).

In order to determine if histone acetylation could affect the endogenous expression of the *CYP46A1* gene, HeLa and SH-SY5Y cells were cultured in the absence, or presence of increasing concentrations of TSA (50 – 250 nM) for 24 h (Figs. 2.2A and B), and for the indicated time-points (Fig. 2.2C). Total RNAs were extracted and CYP46A1 mRNA levels were quantified by real-time PCR (qPCR). In untreated HeLa cells, CYP46A1 mRNA levels could not be determined. Nevertheless, upon TSA treatment, we could measure a dose-dependent increase in CYP46A1 mRNA accumulation (Fig. 2.2A). Similarly, treatment of SH-SY5Y cells with increasing concentrations of TSA also revealed a significant dose-dependent induction of CYP46A1 mRNA levels, of about 55-fold with 250 nM TSA (Fig. 2.2B). Afterwards, we analyzed the kinetics of CYP46A1 mRNA accumulation in SH-SY5Y treated with 250 nM TSA. Our results showed that mRNA accumulation started 3 h after TSA treatment (10-fold over control), and significantly increased to 300-fold over control, 16 h after drug administration (Fig. 2.2C). The effect of other HDAC inhibitors on CYP46A1 mRNA accumulation was also assessed, and qPCR results demonstrated that treatment of SH-SY5Y with 1 mM of VA or NaB for 16 h increased CYP46A1 mRNA levels to 8- and 16-fold over control levels, respectively (Fig. 2.2C). In contrast, the expression of the human β-actin gene was not affected (data not shown). To ensure that the different HDAC inhibitors were inducing an increase in overall acetylation of histone 3 (AcH3) and histone 4 (AcH4), SH-SY5Y were treated with 250 nM TSA, 2 mM VA and 2 mM NaB, for 6 h, and the degree of histone acetylation was assessed by immunoblotting with

antibodies specific for acetylated H3 and H4. As expected, treatment of SH-SY5Y with the HDAC inhibitors resulted in hyperacetylation of H3 and H4 (Fig. 2.2D).

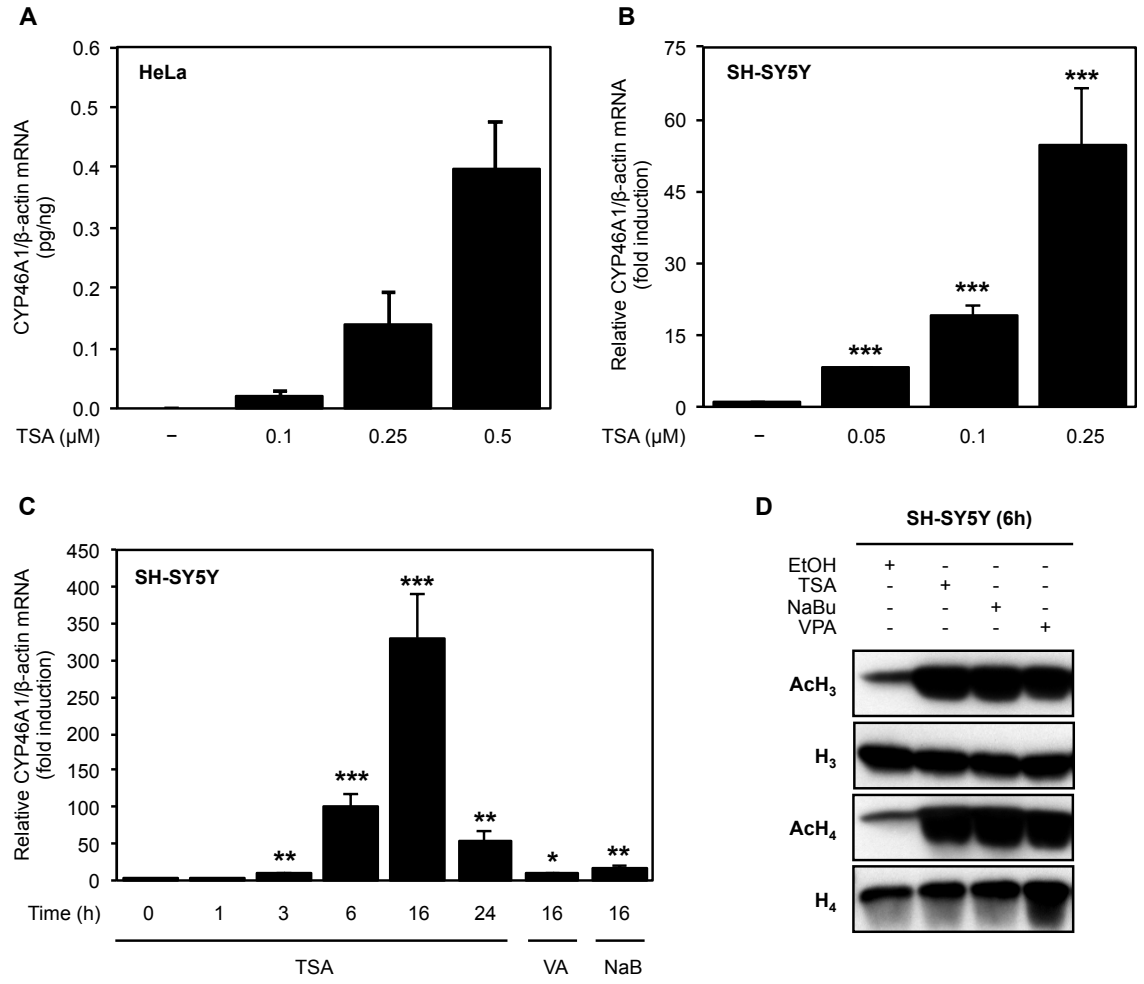

Figure 2.2. TSA induces CYP46A1 mRNA in human neuroblastoma cells. Real-time PCR analysis of CYP46A1 steady-state mRNA transcript levels in HeLa (A) and SH-SY5Y (B) cells treated with the indicated doses of TSA, for 24 h, with 250 nM TSA for the indicated time points (C) and with 1 mM of valproic acid (VA) or 1 mM of sodium butyrate (NaB), for 16 h (C). Values were normalized to the internal standard β-actin. Data represent means ± SEM and were expressed as pg of CYP46A1 mRNA per ng of β-actin mRNA (A), or as relative fold change relative to vehicle-treated cells, that were used as calibrators (B and C) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Global acetylation status of H3 and H4 in SH-SY5Y cells treated with HDAC inhibitors (D). Western blot analysis of histone extracts from SH-SY5Y cells treated with 250 nM TSA, 2 mM VA or 2 mM NaB, for 6 h, was performed for AcH3 and AcH4, and total H3 and H4 were used as loading controls, respectively. Blots are representatives of three independent experiments.

These studies have demonstrated that *CYP46A1* promoter activity and gene transcription were markedly induced when histone deacetylase activity was inhibited, and

that histone deacetylation is prone to be responsible for the significant repression of the *CYP46A1* gene transcription in the tested continuous cell lines.

2.4.2. Sp protein binding is indispensable for TSA-mediated activation of the *CYP46A1* promoter

We have previously shown that the highly GC-rich *CYP46A1* promoter harbored four Sp-responsive elements required and sufficient for high levels of promoter activity (Milagre *et al.* 2008). In order to identify if sites Sp-RE-A, Sp-RE-B, Sp-RE-C and Sp-RE-D were involved in the TSA-mediated induction of the *CYP46A1* promoter, *wild type* promoter (0.12pGL2) and its various mutant constructs were transfected into SH-SY5Y cells, treated with or without 250 nM TSA (Fig. 2.3).

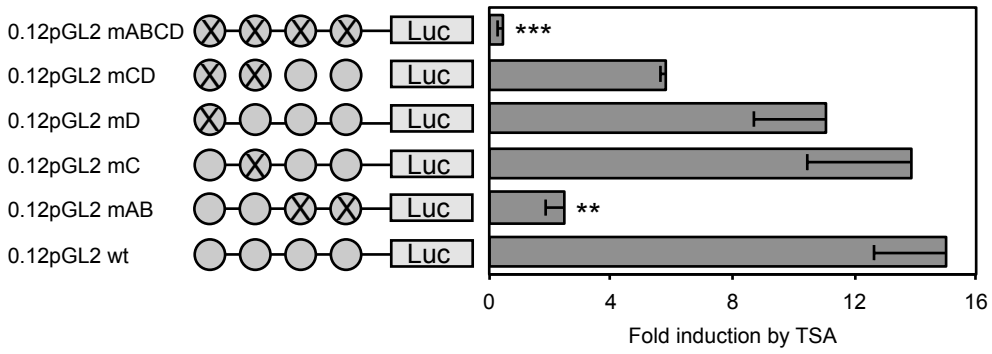

Figure 2.3. Proximal Sp-RE sites are critical for TSA-mediated *CYP46A1* promoter activation. The *wild type* CYP46A1 promoter construct 0.12pGL2 and its various mutants (X represents mutation of the Sp-RE element) were transiently expressed in SH-SY5Y cells. Sixteen hours after transfection, the cells were treated with or without 250 nM TSA for 24 h. Normalized luciferase activities were expressed as mean values of fold increase in the presence of TSA over that observed in the absence of TSA for each constructs ± SEM of duplicates for a minimum of three experiments (** $p < 0.01$; *** $p < 0.001$).

As shown in Fig. 2.3 combined mutation of Sp-RE sites significantly reduced TSA-induced *CYP46A1* promoter activity (ANOVA one-way test: $F = 32.19$, $df = 8$, $p < 0.001$). The *post-hoc* comparisons revealed that significant differences in the fold activation levels were found between the 0.12pGL2 plasmid and the construct bearing combined mutation of Sp-RE-A and Sp-RE-B (Tukey HSD for unequal N, $p < 0.01$), reducing TSA activation to approximately 15% of the *wild type*, while combined

mutation of Sp-RE-A, Sp-RE-B, Sp-RE-C and Sp-RE-D, completely abolished TSA induction (Tukey HSD for unequal N, $p < 0.01$). These results suggest that Sp-RE-A and Sp-RE-B sites are critical for activation/derepression of the *CYP46A1* gene transcription by the HDAC inhibitor TSA.

In order to assess if Sp proteins are indispensable for TSA activation of *CYP46A1* promoter, we used *Drosophila* SL2 cells that lack endogenous Sp factors, allowing the investigation of gene regulation without the interference of endogenous Sp proteins (Hagen *et al.* 1995). The 0.12pGL2 plasmid was transfected into SL2 cells that were treated with or without TSA. In parallel Sp1, Sp3 and Sp4 transcription factors were over-expressed (Fig. 2.4). Our results showed that *CYP46A1* promoter is not responsive to TSA in the absence of Sp transcription factors. Overexpression of the ubiquitously expressed Sp1 or Sp3, or the brain-enriched Sp4 renders the *CYP46A1* promoter responsive to TSA. Nevertheless, although promoter activity was significantly increased by TSA in the presence of Sp proteins, the transactivating effect of TSA in SL2 cells is weaker than in mammalian cell lines (3-fold *vs* 10-fold).

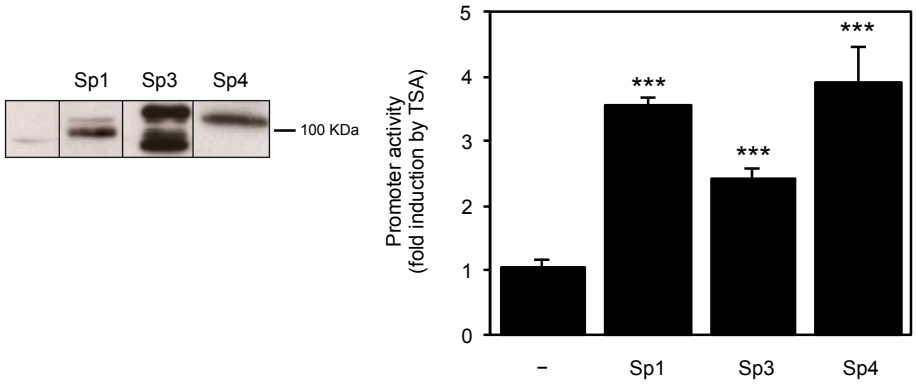

Figure 2.4. Over-expression of Sp proteins, in Sp-deficient *Drosophila* SL2 cells, is required for TSA-induced *CYP46A1* promoter activation. Cotransfection of 0.5 µg of 0.12pGL2 reporter construct with empty vector or with 0.025 µg of pPacSp1, 0.025 µg pPacSp3 or 0.5 µg of pPacSp4 expression vectors in *Drosophila* SL2 cells. Twenty-four hours after transfection, cells were incubated with 500 nM TSA for 24 h. Relative promoter activity is indicated as fold induction over the promoter activity in the absence of treatment. Normalized luciferase activities were expressed as mean values ± SEM of duplicates for a minimum of three independent experiments (*** $p < 0.001$). Result of a representative western blot analysis of Sp proteins in the co-transfection experiments with empty vector (-) or with pPac Sp1FL-compl., pPac-Sp3FL-New or pPac-HD-FLAG-Sp4, is shown.

The requirement of Sp binding sites in TSA-mediated induction of *CYP46A1* promoter activity prompted us to further study the involvement of these transcription factors, by transfecting SH-SY5Y cells with pools of three specific siRNAs designed to reduce the levels of each Sp protein. Indeed, specific knock down of Sp protein levels (Figs. 2.5A and B), significantly prevented TSA-induction of *CYP46A1* gene transcription (ANOVA one-way test: $F = 5.28$, $df = 3$, $p < 0.05$) (Fig. 2.5C). The *post-hoc* comparisons revealed that significant differences in *CYP46A1* transcriptional activation by TSA were found between cells transfected with scrambled siRNA and cells where Sp1 and Sp4 had been silenced (Tukey HSD for unequal N, $p < 0.05$). In contrast, suppression of Sp3 protein had no statistically significant effect on *CYP46A1* response to TSA.

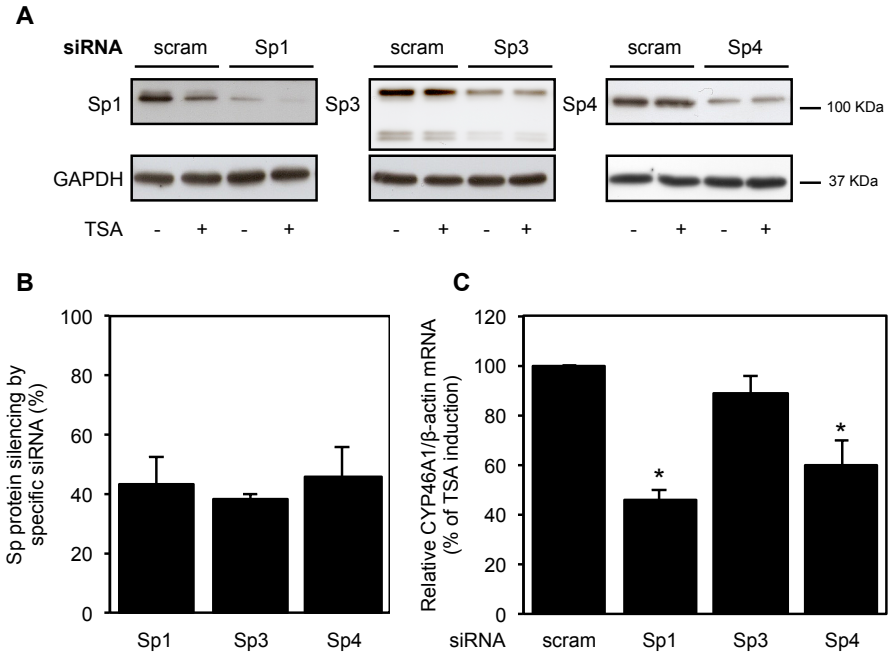

Figure 2.5. Sp proteins are critical for TSA-induced *CYP46A1* gene activation. SH-SY5Y cells were transfected with Sp1, Sp3, Sp4 or scrambled (scram) siRNA, and 56 h after transfection cells were treated with or without 250 nM TSA for additional 16 h. A) Western blot analysis of Sp1, Sp3 and Sp4 protein levels. The level of GAPDH is shown as a loading control. B) Quantification of the relative levels of each Sp proteins after transfection with specific pools of siRNAs. The Sp protein levels in cells transfected with scram siRNA was arbitrarily set as 100%, and the decrease in protein levels was calculated and plotted as a percentage of this value. Data shown are mean values ± SD of at least three independent experiments. C) Quantitative real-time PCR analysis of CYP46A1 mRNA. Values were normalized to the internal standard β-actin. The data were expressed as fold change relative to vehicle-treated cells that were used as calibrators. Fold change obtained in cells transfected with scram siRNA after treatment with TSA was set to 100%, and the effect of silencing each Sp protein was calculated and plotted as a percentage of this value. Blots are representative of three independent experiment and data represent means ± SEM of at least three independent experiments (* $p < 0.05$).

Taken together, these studies demonstrated that the Sp proteins are critical for activation/derepression of *CYP46A1* gene transcription by the HDAC inhibitor TSA.

2.4.3. Recruitment of protein complexes to the *CYP46A1* promoter after histone deacetylase inhibition by TSA

Afterwards we have investigated whether Sp-RE site-dependent activation of the *CYP46A1* promoter activity could be attributed to changes in the different Sp protein binding activities to these response elements in the *CYP46A1* proximal promoter, or to the formation of novel DNA-protein complex(es) after TSA treatment. We have previously characterized that the four Sp-RE sites in the *CYP46A1* proximal promoter were able to bind to each of the different Sp proteins (Milagre *et al.* 2008). As our results suggested that Sp-RE-A and Sp-RE-B sites are critical for activation/derepression of the *CYP46A1* gene transcription by the HDAC inhibitor TSA, we have incubated nuclear extracts of SH-SY5Y cells treated with 250 nM TSA for different time points, with the Sp-RE-B probe, since we have previously demonstrated that Sp proteins have a higher affinity for Sp-RE-B site. The EMSA results showed that the total nuclear proteins capable of binding to the Sp-RE-B probe markedly decreased 4 h after TSA treatment, and then gradually increased to control levels, 24 h after treatment (Fig. 2.6A). To determine the protein composition of these complexes, antibodies against Sp1, Sp3 and Sp4 were added to the DNA-binding assay. Our results showed that the composition of the complexes formed with nuclear extracts from control cells did not differ significantly from those formed with nuclear extracts prepared from cells treated with 250 nM of TSA for 4 h (Fig. 2.6B). We have used antibodies against the different Sp proteins, that we had previously proved to work in supershift analysis (Milagre *et al.* 2008) and our results revealed that Sp3 was the major component of these complexes.

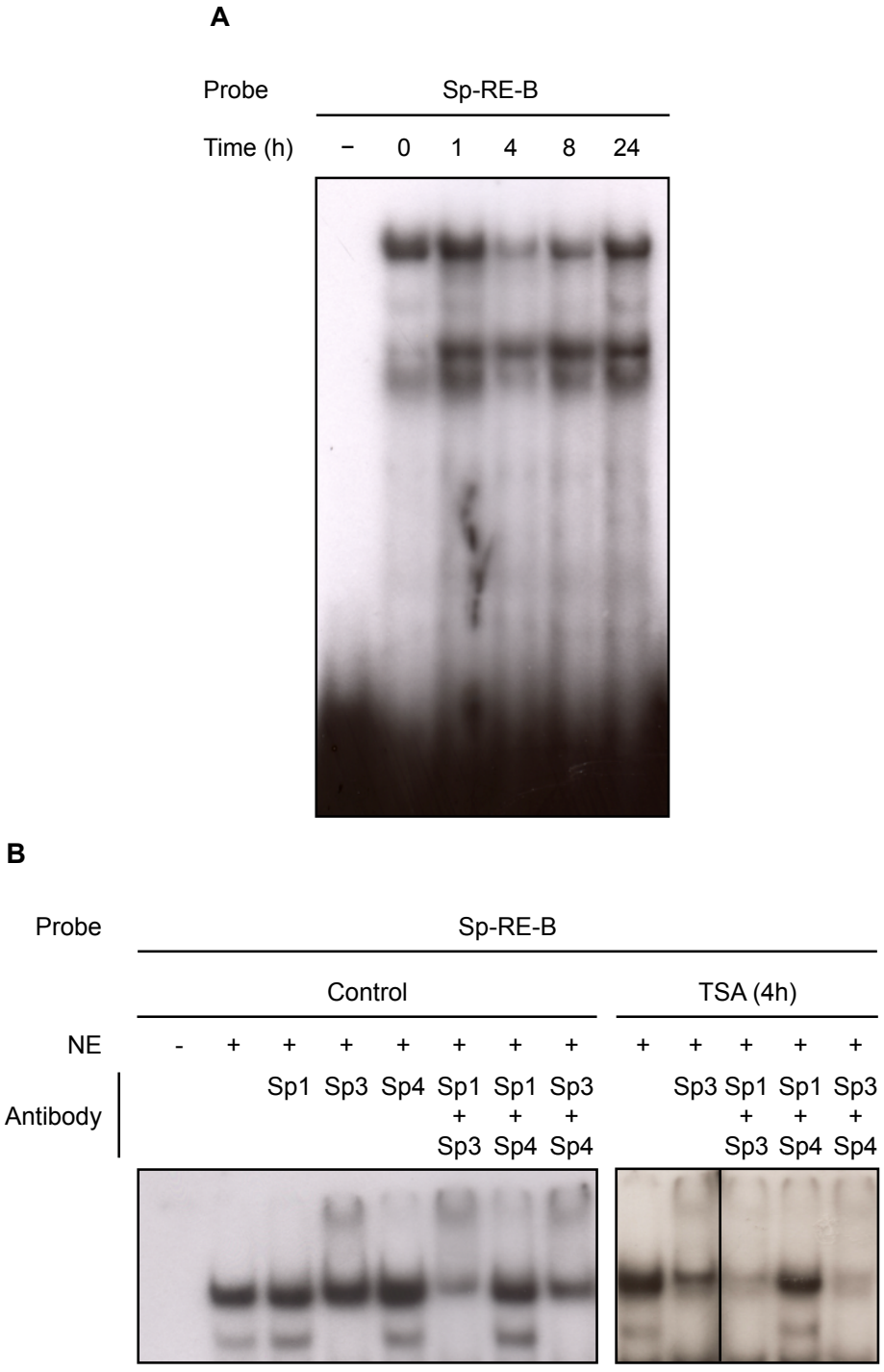

Figure 2.6. TSA affects binding of Sp proteins to the SP-RE-B site of the *CYP46A1* proximal promoter in human neuroblastoma cells. A) EMSA was performed using nuclear extracts of SH-SY5Y cells treated with or without 250 nM TSA for the indicated time points, and a radiolabeled double-stranded oligonucleotide corresponding to the Sp-RE-B site as a probe. B) Super-shift experiments were performed using nuclear extracts (NE) of SH-SY5Y cells treated without or with 250 nM TSA for 4 h, and with anti-Sp1, -Sp3 and -Sp4 antibodies. The autoradiography exposure times for left and right panels were 24 and 48 h, respectively. Autoradiograms are representative of three independent experiments.

Decreased binding of Sp proteins to Sp-RE-B observed after TSA treatment suggested that TSA might be down-regulating Sp proteins expression, or affecting Sp protein activity. Immunoblotting experiments with nuclear extracts from SH-SY5Y cells treated with or without 250 nM TSA for the indicated time points showed that the relative protein levels of Sp1, Sp3, Sp4, HDAC1 and HDAC2 were not significantly affected by TSA treatment (Fig. 2.7A). These results suggested that TSA induced a decrease in Sp protein activity, and that Sp proteins were possible candidates to target the non-DNA-binding HDACs to the *CYP46A1* promoter. In order to further analyze the multiprotein complexes formed in response to TSA, at the *CYP46A1* proximal promoter, we have performed DAPA with a biotin-end-labeled probe corresponding to the promoter sequence that encompasses Sp-RE-A and Sp-RE-B sites. Analysis of precipitated proteins by western blotting revealed that TSA caused a significant detachment of Sp3 from the GC-rich region of the *CYP46A1* proximal promoter. This decrease in binding could be observed 6 h after TSA treatment (Figs. 2.7A and B), and was consistent with our EMSA results. Our results also showed that two histone deacetylases, HDAC1 and HDAC2, were specifically pulled-down by the Sp-responsive region, and that TSA diminished the recruitment of HDAC1 to the *CYP46A1* promoter. Decreased binding of HDAC1 was apparent 6 h after TSA treatment, returning to control levels 12 h afterwards (Figs. 2.7A and B). Release of HDAC1 from the *CYP46A1* proximal promoter was concomitant with the decrease in Sp3 binding, previously described.

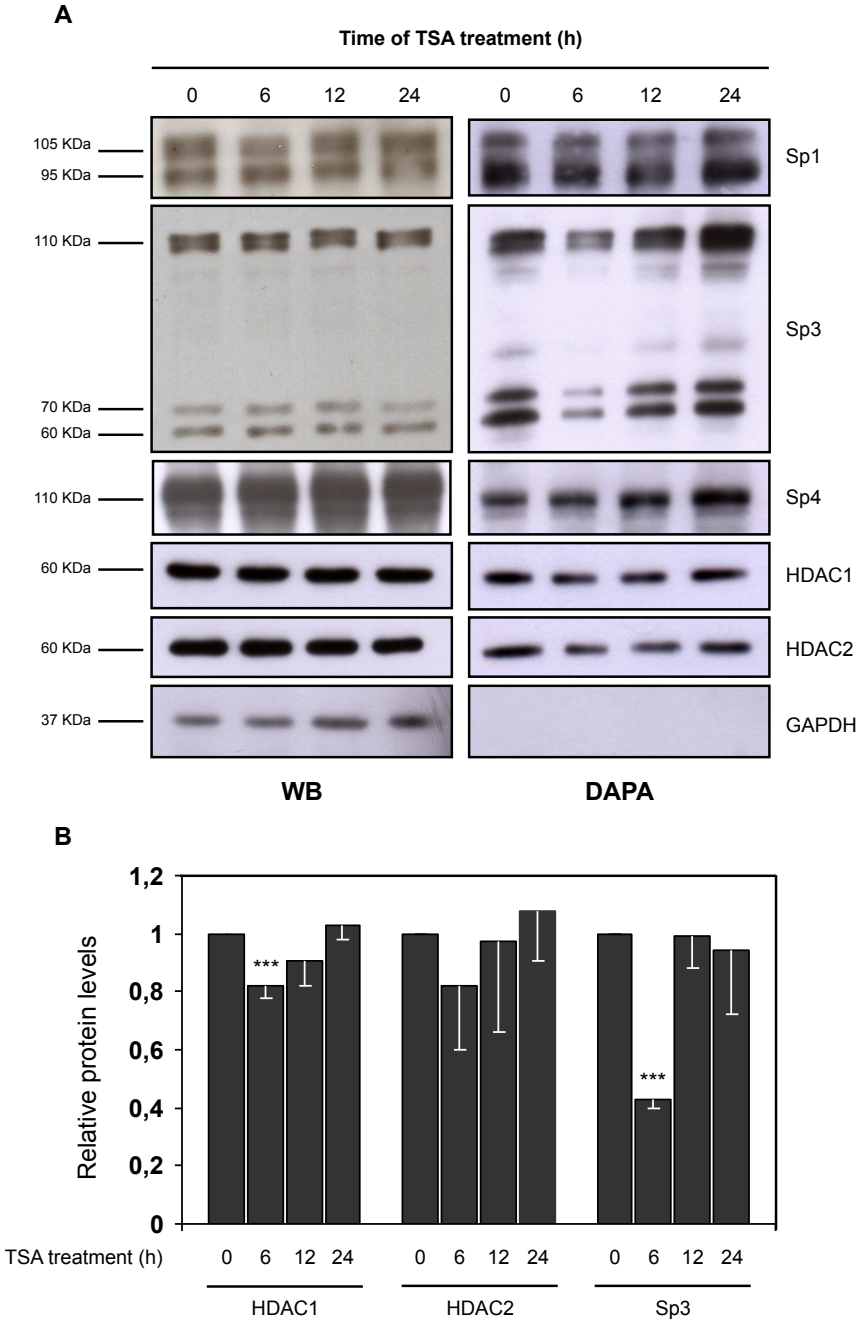

Figure 2.7. TSA induces the release of Sp3 and HDACs from the GC-rich region of the *CYP46A1* proximal promoter. A) Western blot analysis of nuclear proteins extracted from cells treated with or without 250 nM TSA for the indicated time points. Membranes were incubated with specific anti-Sp1, -Sp3, -Sp4, -HDAC1, -HDAC2 and -GAPDH antibodies. DAPA showing the modulation of protein components that bind to the CYP46A1 proximal promoter in response to TSA in SH-SY5Y cells. Biotinend- labeled PCR product corresponding to the sequence -152 to -64, which encompasses sites Sp-RE-A and Sp-RE-B, was incubated with nuclear extracts from cells treated with or without 250 nM TSA for the indicated time points. Precipitated proteins were immunoblotted with the formerly described antibodies. The results shown are representative of those obtained in three independent cultures. B) Quantification of HDAC1, HDAC2 and Sp3 precipitated proteins levels. Results are presented has fold induction over protein levels detected in the absence of TSA treatment. Data shown are mean values ± SD of at least three independent experiments (*** $p < 0.001$).

We next determined the effect of TSA on the association of multiprotein complexes to the *CYP46A1* promoter region using ChIP assays (Figs. 2.8 and 2.9). As our data indicated that Sp transcription factors were required for TSA activation of the *CYP46A1* promoter (Figs. 2.3, 2.4 and 2.5), the binding status of these proteins to the promoter region was determined. Since the distance between the several Sp-RE sites in the GC-rich proximal promoter (ten putative Sp-binding sites in the region encompassed by nucleotides -417 to -64) is smaller than average chromatin fragments produced during sonication (500-900 bp), and no suitable primers can be chosen to discriminate between binding of Sp proteins to the different binding sites, we have designed two sets of primers targeting the +1 region and a more distal promoter region (-1Kb), to detect Sp binding to the *CYP46A1* promoter. Additionally, one set of primers was used to amplify a distal upstream region (-11kB) (Fig. 2.8A).

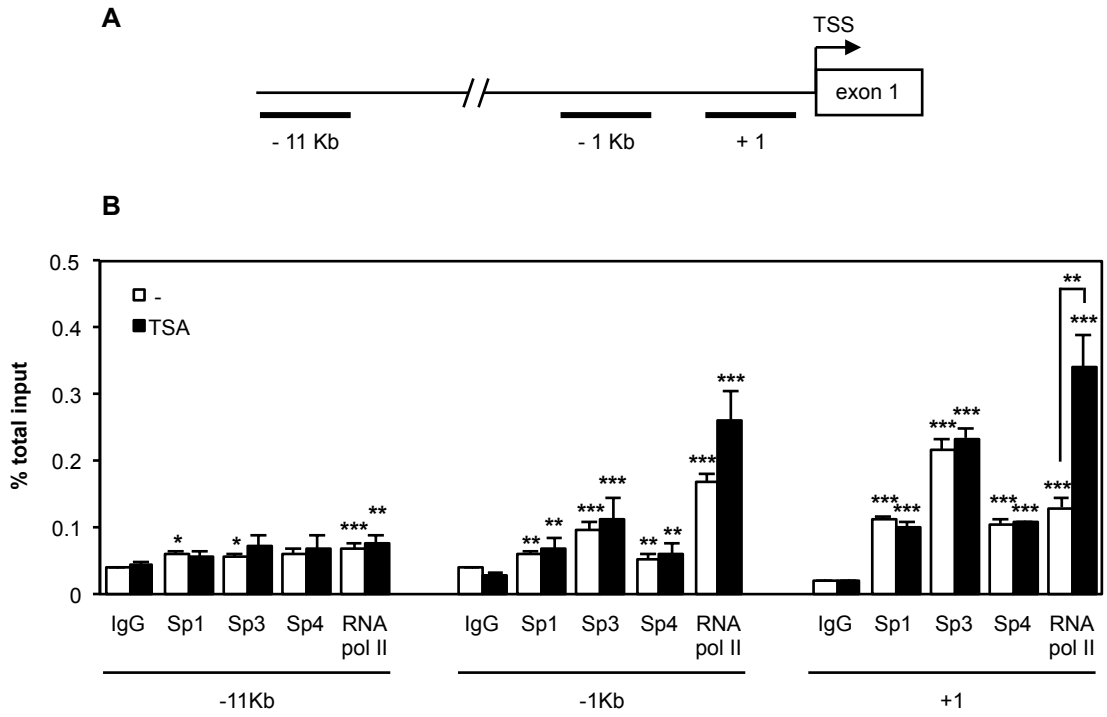

Figure 2.8. Recruitment of Sp transcription factors to the GC-rich region of the *CYP46A1* proximal promoter after TSA treatment. A) Schematic representation of the CYP46A1 promoter and of the real-time amplified regions analysed by ChIP. B) Chromatin from SH-SY5Y was prepared 0 and 6 h after treatment with 250 nM of TSA and immunoprecipitated with anti-Sp1, -Sp3, -Sp4, and -RNA pol II antibodies. After DNA recovery, the precipitates were evaluated by real-time PCR as described in Experimental Procedures. All results are expressed as percentage of total input and represent means of at least three independent experiments ± SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

In untreated SH-SY5Y cells, Sp1, Sp3 and Sp4 were present in the +1 region, supporting a role of these proteins in regulating basal expression of the *CYP46A1* gene (Fig. 2.8B). Unexpectedly, in contrast with our EMSA and DAPA results, TSA treatment of SH-SY5Y cells did not affect the binding of any of the Sp transcription factors to the GC-rich proximal promoter region of the *CYP46A1* gene (Fig. 2.8B), suggesting that although activation of *CYP46A1* transcription by TSA requires Sp binding, it does not induce an overall change in Sp transcription factors binding to this region of the promoter. Nevertheless, following treatment with TSA, RNA polymerase II (RNA pol II) binding was significantly increased in the +1 promoter region, by 160%, which likely triggers the observed increase in *CYP46A1* gene transcription (Figs. 2.8B and 2.2B and C).

Next, we examined the binding of HDACs and HATs to the *CYP46A1* promoter. In agreement with our DAPA results, HDAC1 and HDAC2 were significantly dissociated from the +1 promoter region, by 35 and 31%, respectively, 6 h after TSA treatment (Fig. 2.9A). Concomitantly, a significant increase of 50% in the recruitment of the coactivator CBP to the +1 region could be observed. Likewise, p300, that was not associated to any region of the *CYP46A1* promoter prior to TSA treatment, could be detected in the -1Kb region, 6 h after HDAC inhibition. The shift in HDACs/HATs equilibrium following treatment with TSA occurs concomitantly with the enrichment of RNA pol II in the promoter region.

ChIP analysis was also used to study histone modifications at the *CYP46A1* promoter (Fig. 2.9). Antibodies specific to diacetylated H3 (K9 and K14), tetra-acetylated H4 (K5, K8, K12 and K16), K4 trimethylation of histone H3 (H3K4me3), and K4 dimethylation of histone H3 (H3K4me2), were used to determine the pattern of histone modifications upon TSA exposure. In untreated cells, both H3 and H4 were acetylated at the -1Kb and +1 region, with the highest enrichment detected at the -1Kb area. Six hours after TSA treatment we could not detect any increase in the association of acetylated H3 and H4 (Fig. 2.9A). Therefore, since histone acetylation has been previously determined as early as 15 min after treatment with HDAC inhibitors (Hazzalin & Mahadevan 2005, Rada-Iglesias *et al.* 2007), we decided to study the kinetics of histone acetylation at the *CYP46A1* promoter (Fig. 2.9B).

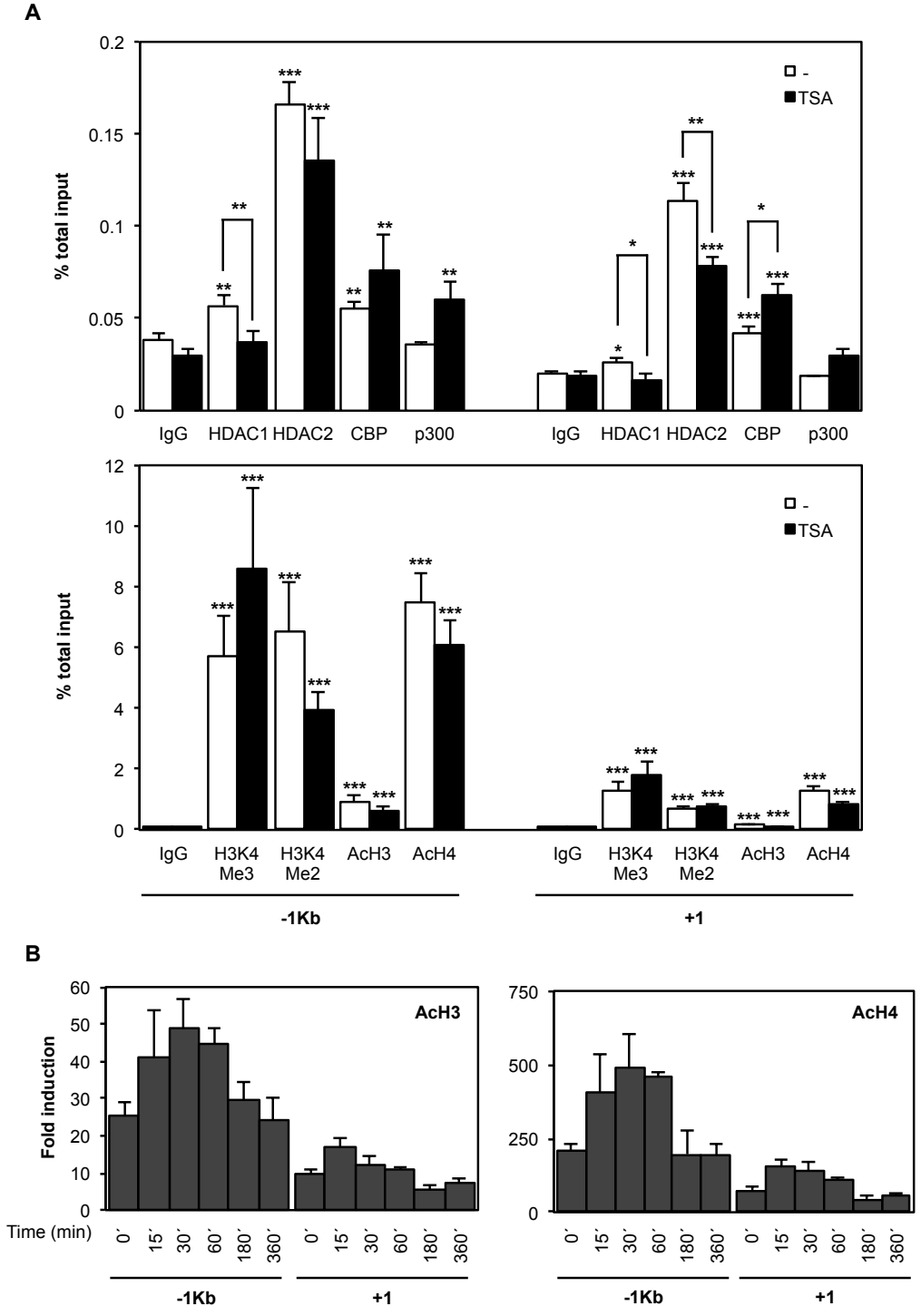

Figure 2.9. TSA affects the recruitment/release of HATs, HDACs and acetylated histones to the GC-rich region of the *CYP46A1* proximal promoter. A) Chromatin from SH-SY5Y was prepared 0 and 6 h after treatment with 250 nM of TSA and immunoprecipitated with anti- HDAC1, -HDAC2, -p300, -CBP, -H3K4me3, -H3K4me2, -acetyl H3 and -acetyl H4. Results are expressed as percentage of total input and represent means of at least three independent experiments ± SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). B) Chromatin from SH-SY5Y was prepared at 0, 15, 30, 60, 180 and 360 min after treatment with 250 nM of TSA and precipitated with antibodies directed against -acetyl H3 and -acetyl H4. After DNA recovery, the precipitates were evaluated by real-time PCR as described in Experimental Procedures. Results are expressed as fold change over IgG and represent means of at least three independent experiments ± SEM.

Hence, we have treated SH-SY5Y cells for 0, 15, 30, 60, 180 and 360 min, and determined that, upon TSA treatment, histone acetylation, increased at 15 min post-induction at the -1Kb region, reached a maximal level at 30 min, remained high at 60 min, and began to drop, reaching control levels 6 h after treatment. Interestingly, a similar kinetic of histone acetylation could be observed at the +1 region, although with a much lower magnitude. Thus, we can conclude that TSA caused a dramatic increase in H4 acetylation, and to a lower extent in H3 acetylation, at early time points (15-30 min), which however is transient. Furthermore, histone deacetylation was already evident 6 h after treatment, at a time point when the HDAC/HAT ratio would still favor acetylation. As for histone methylation, we could observe that the region with higher levels of acetylated histones (-1Kb) displayed also higher levels of H3K4me3, a well-known active gene marker (Kouzarides 2007) (Fig. 2.9A). We could also observe, in SH-SY5Y cells treated with TSA for 6 h, an increase in the recruitment of H3K4me3, which was concomitant with a decrease in H3K4me2, although this trend did not reach statistical significance (Fig 2.9A).

Taken together, these results indicate that induction of *CYP46A1* by TSA is correlated with a shift of HDAC/HAT equilibrium and a concomitant dynamic change in the chromatin structure, with an increase in the local recruitment of euchromatic markers that lead to the recruitment of RNA pol II, which likely triggers the observed increase in *CYP46A1* gene transcription (Fig. 2.8).

2.5. Discussion

In this work we have investigated if *CYP46A1* gene expression might be regulated by epigenetic mechanisms. The *CYP46A1* proximal promoter is located within a CpG island, suggesting that *CYP46A1* expression might be regulated by DNA methylation. Nevertheless, our preliminary bisulphite sequencing data have shown that the *CYP46A1* proximal promoter is completely demethylated in different human tissues (unpublished results). Therefore, we have focused on the role of histone modifications in the control of *CYP46A1* gene transcription.

The present study has demonstrated that inhibition of HDAC activities by TSA, VA and NaB caused a potent induction of human *CYP46A1* expression, in agreement

with the results published by Shafaati and co-workers, that demonstrated a marked time-dependent derepression of the expression of *Cyp46a1* in mice, in response to treatment with TSA (Shafaati *et al.* 2009). Furthermore, we have shown that Sp protein binding to the GC-rich region in the proximal promoter, which we have previously characterized to be essential for basal promoter activity, is also critical for TSA response. GC-rich sequences have been previously implicated in tissue-specific gene expression, but also in the control of transcription in response to different stimuli. For instance, the cell cycle protein $p21^{Waf1/Cip1}$ is regulated by NaB (Nakano *et al.* 1997), TSA (Sowa *et al.* 1997), apicidin (Kim *et al.* 2003), nerve growth factor (Yan & Ziff 1997) and transforming growth factor β (Datto *et al.* 1995) *via* GC-rich sequences within its promoter. The function of GC-rich sequences usually involves the ubiquitously expressed Sp family of proteins, which are transcription factors that bind GC-rich and related GA- and GT-boxes (reviewed by Li *et al.* 2004), and regulate several constitutively active or inducible genes. Indeed, an increasing number of studies have pointed to Sp1/Sp3 multiprotein complexes as mediators of histone deacetylase inhibition effect on gene activation (Huang *et al.* 2005, Zhang & Dufau 2002, Zhao *et al.* 2003, Amin *et al.* 2007, Kim et al. 2003).

Although our studies revealed that proximal Sp binding sites are essential for activation of *CYP46A1* by TSA, the different Sp proteins seem to have different critical roles in TSA-induced *CYP46A1* gene activation. Indeed, TSA-induced CYP46A1 mRNA accumulation was only significantly reduced by suppression of Sp1 and Sp4 expression. On the other hand, TSA led to a decrease in Sp3 binding to the SP-RE-B site, 4-8 h after treatment, as shown by the EMSA and DAPA results. This decrease in Sp3 binding to the Sp-RE-B could not be confirmed by ChIP analysis, probably because it is impossible to discriminate between binding of Sp proteins to the different binding sites, as formerly mentioned. Nevertheless, ChIP analysis has revealed that, although TSA treatment did not induce an overall change in the Sp transcription factors binding to *CYP46A1* proximal promoter, activation induced by HDAC inhibitors required Sp proteins binding to this proximal region. These results are in agreement with previous observations that Sp transcription factors constitutively associate with its cognate binding sites on several gene promoters induced by HDAC inhibitors, although no significant differences were found in the association to the promoters (Kim *et al.* 2009, Kim *et al.* 2003, Zhang & Dufau 2002, Zhao *et al.* 2003).

The differential participation of Sp1 and Sp3 proteins in HDAC inhibition-mediated effects has been previously reported, and probably may depend on the cell system employed, and/or to the mechanism involved in the activation. Sp1 but not Sp3 has been reported to mediate HDAC inhibitor response of the luteinizing hormone receptor (Zhang *et al.* 2006), endothelial nitric-oxide synthase (Gan *et al.* 2005) and nonsteroidal anti-inflammatory drug-activated gene (Yoshioka *et al.* 2008). In contrast, Sp3 but not Sp1 mediate TSA response of BAK (Chirakkal *et al.* 2006), and Na^+/H^+ exchanger gene (Kiela *et al.* 2007). Interestingly, Sp3 but not Sp1 is responsible for mediating the TSA induction of $p21^{WAF1/Cip}$ gene in MG63 cells (Sowa *et al.* 1999), while both proteins are involved in the regulation of the same gene by TSA in NIH3T3 cells (Xiao *et al.* 1999).

ChIP analysis demonstrated that a shift in HDAC/HAT equilibrium occurred following treatment with TSA. Indeed HDAC1 and HDAC2 were significantly dissociated from the promoter region, while a significant increase in the recruitment of the coactivators p300/CBP could be observed. Sp3 has been demonstrated to physically interact with specific repressor complexes, namely with HDACs and mSin3A (Zhang & Dufau 2002, Clem & Clark 2006, Sun *et al.* 2002). The decrease in Sp3 binding at particular responsive elements, namely at the Sp-RE-B site, can locally shift the Sp1/Sp3/Sp4 ratio, and favor the detachment of HDAC1 and HDAC2 and the recruitment of HATs. Loss of Sp3 binding, that might be required for HDAC association, may result in the recruitment p300/CBP to the promoter, leading to basal acetylation of the histone tail and maintenance of the open configuration of the local chromatin. Furthermore, such events occurring at the proximal promoter provide chromatin accessibility to RNA polymerase II to initiate the transcription of the *CYP46A1* gene. A large number of studies have reported Sp1 and p300 interaction (Suzuki *et al.* 2000), and cooperation to transactivate the expression of several genes, namely 12(s)-lipoxygenase (Hung *et al.* 2006), and TβRII (Huang *et al.* 2005).

ChIP analysis of histone acetylation has shown that TSA caused a dramatic but transient increase in H3 and H4 acetylation, at early time points (15-30 min), at the promoter region. Furthermore, histone deacetylation was already visible 6 h after TSA treatment, at a time point when the HDAC/HAT ratio would still favor acetylation. This result suggested that TSA provokes an initial increase in histone acetylation, followed by increased transcription that at longer times may be accompanied by post-transcriptional

mechanisms once histone acetylation decreases to its basal level. Our results are in agreement with results reported by Hazzalin and Mahadevan that showed that histone hyperacetylation, at gene promoters after HDAC inhibitor treatment, is an early and transient event. In fact, acetylation of H3 around the transcription start site of the inducible proto-oncogenes *Fos* and *Jun* has been described to be rapidly increasing, peaking at 15 min to 1 h after TSA treatment (Hazzalin & Mahadevan 2005). However, after a 2-h treatment, levels returned to almost basal and after 4 h, histone deacetylation could be observed, when compared to controls.

Our results have also shown that the +1 region, that we expected to be the most prominent area in histone modifications, showed only modest alteration, and the strongest enrichment was found in the -1kb region. Furthermore, our results showed that the region that displayed higher levels of acetylated histones (-1Kb), also displayed higher levels of H3K4me3, and that H3K4me3 histones could be found in the promoter of untreated cells. It has been suggested that conservation of H3K4me3 and perhaps of other "active" marks, could help in the recruitment of HATs and could render promoter regions in a sufficiently accessible state, allowing pre-initiation complex and some transcription factors to remain bound at core promoters (Rada-Iglesias *et al.* 2007). Co-localization of H3 acetylation directly with K4 methylation at the same regions of the genome and on the same H3 tail has been previously reported (Hazzalin & Mahadevan 2005, Song & DeBose-Boyd 2004). Moreover, Bradbury and colleagues, using mass spectrometry, showed that H3 tails that are K4-methylated are also likely to be hyperacetylated (Zhang *et al.* 2004).

Altogether, our data also indicated that although *CYP46A1* is expressed at very low levels in neuroblastoma cells, the promoter conserves some of its transcriptionally active "landscape", which might explain why the TSA effect was virtually instantaneous, detectable by immediate localized histone acetylation.

The recent report of Hudry and co-workers, demonstrating that *in vivo* overexpression of CYP46A1 before and after the onset of amyloid plaques significantly reduces amyloid β pathology in mouse models of Alzheimer's disease (Hudry *et al.* 2010), highlights the importance of identifying molecules that can modulate *CYP46A1* expression. Up to now, HDAC inhibitors are the only compounds known to up-regulate the expression of this gene. Moreover, studies on CYP46A1 knockout-mice also suggest its importance in memory function (Kotti *et al.* 2006), emphasizing once more the importance of understanding the molecular mechanisms underlying *CYP46A1* induction

by HDAC inhibitors. Interestingly, HDAC inhibitors have recently been shown to induce sprouting of dendrites, increase number of synapses, and reinstate learning behaviour and access to long-term memories (Fischer *et al.* 2007).

In summary, this study shows that HDAC inhibition increases *CYP46A1* gene expression, and that multiple layers of regulation are necessary to attain maximal activation of CYP46A1 gene transcription, namely histone hyperacetylation, and a shift in the Sp1/Sp3/Sp4 ratio, that favours the dissociation of HDACs and the recruitment euchromatin markers.

2.6. Acknowledgments

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CHAPTER 3

OKADAIC ACID INHIBITS THE TRICHOSTATIN A-MEDIATED INCREASE OF HUMAN *CYP46A1* NEURONAL EXPRESSION IN A ERK1/2-SP3-DEPENDENT PATHWAY

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3.1. Abstract

The *CYP46A1* gene codes for the cholesterol 24-hydroxylase, a cytochrome P450 specifically expressed in neurons, and responsible for the majority of cholesterol turnover in the central nervous system. Previously, we have demonstrated the critical participation of Sp transcription factors in the *CYP46A1* response to HDAC inhibitors and in this study we investigated the involvement of intracellular signaling pathways in the trichostatin A (TSA) effect. Our results show that pre-treatment of neuroblastoma cells with chemical inhibitors of MEK1 significantly potentiates the TSA-dependent induction of *CYP46A1*, while inhibition of protein phosphatases by okadaic acid (OA), or overexpression of MEK1, partially impairs the TSA effect without affecting histone hyperacetylation at the promoter. Immunoblotting revealed that TSA treatment decreases ERK1/2 phosphorylation concomitantly with a decrease in Sp3 binding activity, which are both reversed by pre-treatment with OA. Chromatin immunoprecipitation analysis demonstrated that TSA induces the release of p-ERK1/2 from the *CYP46A1* proximal promoter, while pre-treatment with OA restores the co-occupancy of Sp3-ERK1/2 in the same promoter fragments. With our work we demonstrate for the first time the participation of MEK-ERK1/2 signaling pathway in HDAC inhibitors-dependent induction of cytochrome P450 gene expression, underlying the importance of this regulatory signaling mechanism in the control of brain cholesterol elimination.

3.2. Introduction

The brain-specific cholesterol 24-hydroxylase (CYP46A1) is a member of the cytochrome P450 superfamily of enzymes that catalyzes the hydroxylation of cholesterol into 24(S)-hydroxycholesterol (24OHC) (Lund *et al.* 1999). The flux of this oxysterol across the blood-brain barrier represents the major mechanism of cholesterol elimination from the brain having, therefore, a crucial role in reverse cholesterol transport and in the maintenance of brain cholesterol homeostasis (Bjorkhem *et al.* 1998). *Cyp46a1* $^{-/-}$ knockout mice exhibit severe deficiencies in spatial, associative and motor learning, and hippocampal long-term potentiation (LTP) (Lund *et al.* 2003), which are related to a 40% reduction in brain cholesterol synthesis (Kotti *et al.* 2006). In fact, the decreased availability of geranylgeraniol, a nonsterol isoprenoid generated by the mevalonate pathway, but not cholesterol, was found to be essential for LTP, suggesting a major role of neuronal cholesterol turnover, and directly of CYP46A1, in processes such as learning and memory (Kotti *et al.* 2006, Kotti *et al.* 2008). Moreover, an increasing number of studies suggest that CYP46A1 affects the pathophysiology of Alzheimer's disease (AD) (Brown *et al.* 2004, Famer *et al.* 2007, Prasanthi *et al.* 2009). A study from Hudry and co-workers highlighted the possibility that CYP46A1 overexpression can be a therapeutic approach in AD, since it significantly reduced the amyloid β pathology in mouse models of the disease, before and after the onset of the amyloid plaques (Hudry *et al.* 2010).

Currently, epigenetic modifiers are the only compounds known to induce *CYP46A1* expression (Shafaati *et al.* 2009, Nunes *et al.* 2010). Characterization of the molecular mechanisms involved in the trichostatin A (TSA)-mediated derepression of *CYP46A1* gene revealed that HDAC inhibition specifically induced histone hyperacetylation of *CYP46A1* promoter, concomitantly with an increase in the recruitment of RNA polymerase (RNA pol) II (Nunes *et al.* 2010). Interestingly, the proximal promoter region, encompassing four Specificity protein-responsive elements (Sp-RE) that we have shown to be indispensable for basal *CYP46A1* promoter activity (Milagre *et al.* 2008, Milagre *et al.* 2012a), is also essential for the TSA-mediated activation. Despite the requirement of Sp proteins binding to this proximal promoter region, for the activation by HDAC inhibitors (HDACi), we have verified that a decrease in Sp3 binding at specific responsive elements is important for the shift in HDAC/histone acetyltransferase (HAT) equilibrium that leads to dynamic changes in chromatin structure

(Nunes *et al.* 2010). Moreover, pre-treatment of neuroblastoma cells with the demethylating agent 5-aza-2-deoxicytidine before TSA treatment significantly potentiates the TSA-mediated *CYP46A1* activation, in a DNA methylation independent mechanism, inducing a decrease in Sp3/HDAC binding to the promoter of this neuronal specific gene (Milagre *et al.* 2010). Nevertheless, the fact that histone deacetylation was already evident 6h after TSA treatment, at a time point when the HDAC/HAT ratio should still favor acetylation, lead us to investigate if other mechanisms, besides histone hyperacetylation could also participate in the TSA-mediated derepression of the *CYP46A1* gene. Since Sp1/Sp3 members of the Sp-family of transcription factors are ubiquitously expressed, post-translational modifications assume a key role in the regulation of their transcriptional activity (Waby *et al.* 2008), and might explain the stimulatory changes induced by the HDAC inhibitors in *CYP46A1* transcription, as already described for other genes (Espinos *et al.* 1999, Camarero *et al.* 2003, Zhang *et al.* 2006, Chen *et al.* 2005). In addition, Sp proteins have been described to recruit histone modifying enzymes and chromatin remodeling complexes to specific gene promoters. Sp1 and Sp3 can recruit Sin3A HDAC1/HDAC2 complex (Zhang & Dufau 2003) or the co-activators CPB/p300 (Ammanamanchi *et al.* 2003), and act, respectively as repressors or activators of transcription.

In the present study we aimed to identify the putative participation of specific signaling pathway(s) in the TSA-mediated activation of the *CYP46A1* gene transcription and further elucidate the molecular mechanisms governing the expression of this brain-specific gene and involved in the control of brain cholesterol homeostasis. We clearly demonstrate the participation of the mitogen activated kinase kinase (MEK) - extracellular signal-regulated kinase-1 (ERK) signaling pathway in the *CYP46A1* derepression by TSA treatment. Modulation of Sp3 binding activity, in a ERK1/2-dependent manner, was identified as a crucial step for the TSA effect, independently of histone hyperacetylation, underlying the importance of this regulatory signaling mechanism in the control of brain cholesterol elimination.

3.3. Methods

3.3.1. Reagents and antibodies

All chemical inhibitors – TSA, OA, H89, U0126, SP600129, PD98059 and Gö6983 – were from Sigma (Sigma Aldrich Inc., St Louis, MO, USA). The antibodies used in this work were: anti-p-ERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), -ERK1/2, -p-JNK and -JNK (Cell Signaling Technology, Danvers, MA, USA) for western blot, and anti-Sp3 (Santa Cruz Biotechnology Inc.), -acetyl-histone H4 and –RNA pol II (Millipore, Bedford, MA, USA) for chromatin immunoprecipitation (ChIP).

3.3.2. Cell culture, reporter gene constructs and transactivation assays

The SH-SY5Y human neuroblastoma cell line was maintained and transiently transfected as previously described (Milagre *et al.* 2008). The different recombinant *wild type* and mutated plasmids, derived from the 5' flanking region of the human *CYP46A1* gene, and used in this work, have also been described previously (Milagre *et al.* 2008). NTERA-2cl.D1 (NT2) testicular embryonal carcinoma cells were cultured and differentiated as formerly described (Milagre *et al.* 2012a).

3.3.3. *CYP46A1* expression analysis

Total cell RNA was extracted using Trizol Reagent (Invitrogen Carlsbad, CA, USA) following manufacturer's instructions. Real-Time PCR (qPCR) analysis for CYP46A1 mRNA levels was performed as previously described (Nunes *et al.* 2010), in an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). Results presented are from at least three individual experiments and each sample was assayed in triplicate. The mRNA levels were normalized to the level of β-actin, and are presented as fold change from controls, using the ΔΔCt method. Statistical analysis was performed using ΔCt values.

3.3.4. Western blot analysis

Cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-X 100) containing 1 mM DTT, in the presence of phosphatase inhibtors (10 mM NaF and 1 mM Na_3VO_4) and a protease inhibitor mixture (Roche Diagnostics GmbH, Penzberg, Germany). After incubation at 4ºC, for 30 min, samples were sonicated four times for 4 seconds each, on ice, followed by centrifugation at 12000 *g* for 15 min. Proteins were subject to SDS-PAGE gels, electroblotted onto Immobilon P (Millipore) and incubated with specific antibodies.

3.3.5. Electrophoretic mobility shift assay

EMSA was performed as previously prescribed (Milagre *et al.* 2008). Briefly, 2-5 μg of nuclear extracts, prepared as described by Schreiber and co-workers (Schreiber *et al.* 1989), were incubated with γ^{32}P-labeled oligonucleotide probe harboring sites SP-RE-B or SP-RE-D. Protein-DNA complexes were resolved on 5% native polyacrylamide gels and visualized by autoradiography.

3.3.6. Chromatin immunoprecipitation (ChIP) and re-ChIP

ChIP assays were performed as described previously (Nunes *et al.* 2010). For the re-ChIP experiments, after the first immunoprecipitaton, the DNA/protein complexes were recovered by incubation with 100 mM DTT for 30 min at 37ºC and subsequently diluted in IP dilution buffer [0.01% SDS, 0.5% Triton X100, 2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 100 mM NaCl]. After dilution, a second immunoprecipitation was performed. The recovered DNA was analyzed by real-time PCR in an ABI7300 sequence detection system (Applied Biosystems) with SYBR green Master Mix (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Primers covering different regions of the *CYP46A1* promoter were used: 5' – GCGGACCTGAGTCTGAAGAG -3' (forward) and 5'-AATCACAACTCCGCTTCTGG-3' (reverse) for the proximal promoter (+1 region), and 5'-GGGAAGCCCTGGTCATTATT-3' (forward) and 5'-GTTGGAGTTGGAGGGATGAA-3' (reverse) for the distal promoter (-1 kb region).

3.3.7. Statistical analysis

Statistical analysis was performed using the Student's t-test and the ANOVA one-way test with the Tukey HSD *post-hoc* test or the Tukey HSD for unequal N (Spjotvoll/Stoline test). The analysis was performed using STATISTICA (data analysis software system), version 9.1 StatSoft, Inc. (2010).

3.4. Results

3.4.1. Inhibition of MEK1 potentiates the TSA-mediated derepression of *CYP46A1* gene

To investigate whether specific signaling pathway(s) were involved in the activation of *CYP46A1* gene by the histone deacetylase inhibitor TSA, SH-SY5Y cells were pre-treated with specific kinase/phosphatase inhibitors for 1h followed by 16 h treatment with 250 nM TSA (Fig. 3.1). To evaluate the *CYP46A1* promoter activity, the 0.12pGL2 reporter promoter construct was used, since we have previously described this region as being critical for the TSA response (Nunes *et al.* 2010). The TSA-mediated increase in *CYP46A1* promoter activity was significantly potentiated in the presence of PD98059, a MEK1 inhibitor, while the inhibition of protein kinase A (H89), protein kinase C (Gö6983) or c-Jun N-terminal kinase (JNK) (SP600125) had no effect on TSA induction (Fig. 3.1A; ANOVA one-way test: $F = 14.57$, df = 3, $p < 0.001$; Tukey HSD for unequal N $p < 0.05$). Conversely, the inhibition of protein phosphatases (PP) by okadaic acid (OA) partially impaired the TSA effect, in a dose-dependent manner (Fig. 3.1A; ANOVA one-way test: $F = 8.421$, df = 2, $p < 0.05$; Tukey HSD for unequal N $p < 0.05$). In accordance, real-time PCR analysis revealed that inhibition of MEK1 by pre-treatment with either PD98059 and U0126, another MEK1 inhibitor, increases the CYP46A1 mRNA levels, in comparison with levels attained in cells treated with TSA alone, while the inhibition of PPs by OA has the opposite effect, resulting in a decrease to approximately 30% of the TSA effect (Fig. 3.1B; ANOVA one-way test: $F = 15.2$, df = 3, $p < 0.001$; Tukey HSD for unequal N $p < 0.001$). Moreover, to assess the specificity of the transcriptional response, we next examined the effect of OA treatment on the reelin

gene expression. Our results showed that OA did not induce any significant changes in the reelin mRNA level of cells treated with OA or TSA plus OA (data not shown).

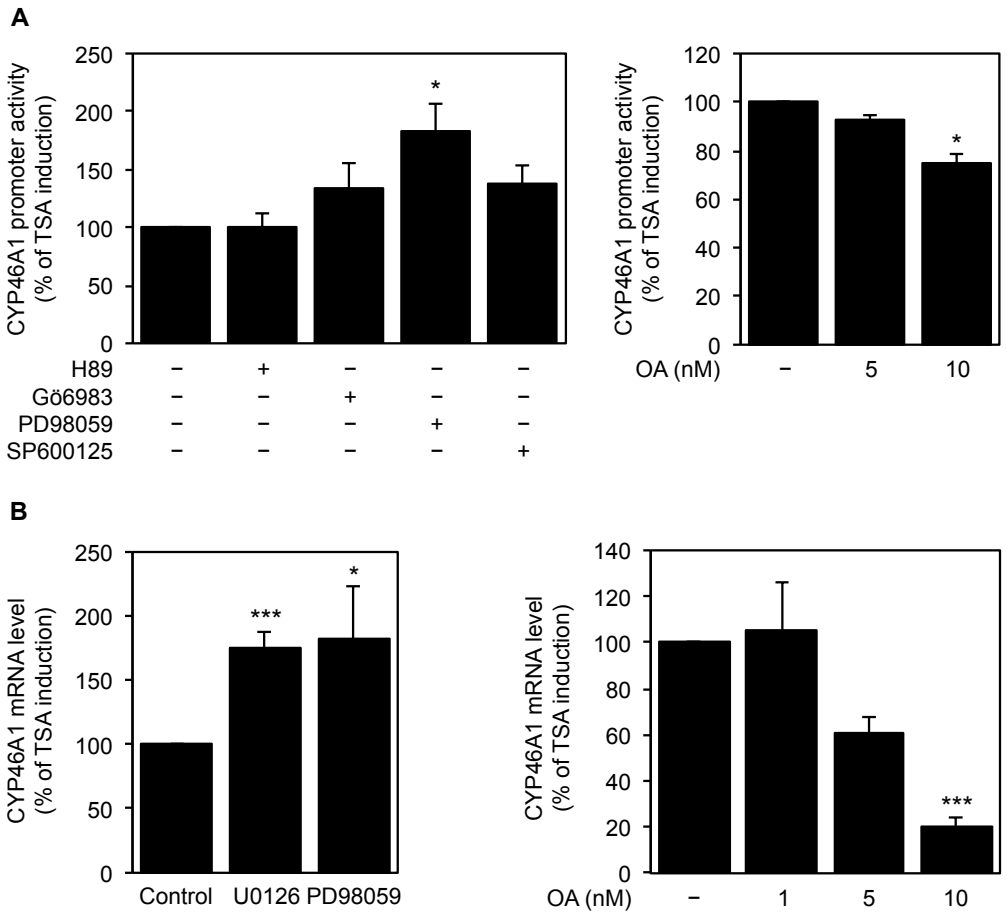

Figure 3.1. Effect of kinase/phosphatase chemical inhibitors on TSA mediated induction of *CYP46A1* expression. A) The 0.12pGL2 plasmid, encompassing the region −236 to −64 of CYP46A1 promoter, was transfected into SH-SY5Y cells. Twenty-four hours after transfection, cells were pre-incubated with 5 μM H89 (PKA inhibitor), 2 μM Go6983 (PKC inhibitor), 10 μM PD98059 (MEK1 inhibitor), 10 μM SP600125 (JNK inhibitor), or with the indicated concentrations of OA for 1 h with or without 250 nM TSA for 16 h. Normalized luciferase activities were expressed as mean values ± SEM of duplicates for a minimum of three independent experiments. B) Real-time PCR analysis of CYP46A1 mRNA transcript levels in SHSY5Y cells pretreated 1 h with 10 μM of the MEK inhibitors U0126 and PD98059 or with the indicated concentrations of OA with or without 250 nM TSA for 16 h. Values were normalized to the internal standard β-actin. Data represent mean values ± SEM of at least three independent experiments and are expressed as percentage of induction relative to TSA-treated cells (* $p < 0.05$; *** $p < 0.001$).

These results clearly demonstrate the participation of phosphorylation/ dephosphorylation events in the HDACi-mediated derepression of *CYP46A1* gene, particularly the involvement of the MEK signaling pathway.

3.4.2. ERK1/2 signaling pathway mediates the increase in *CYP46A1* expression by TSA

To confirm the participation of the MEK-ERK signaling pathway in the TSA-mediated derepression of *CYP46A1* gene, overexpression of both the *wild type* and mutated forms of ERK1 and 2 was performed in SH-SY5Y cells (Fig. 3.2A).

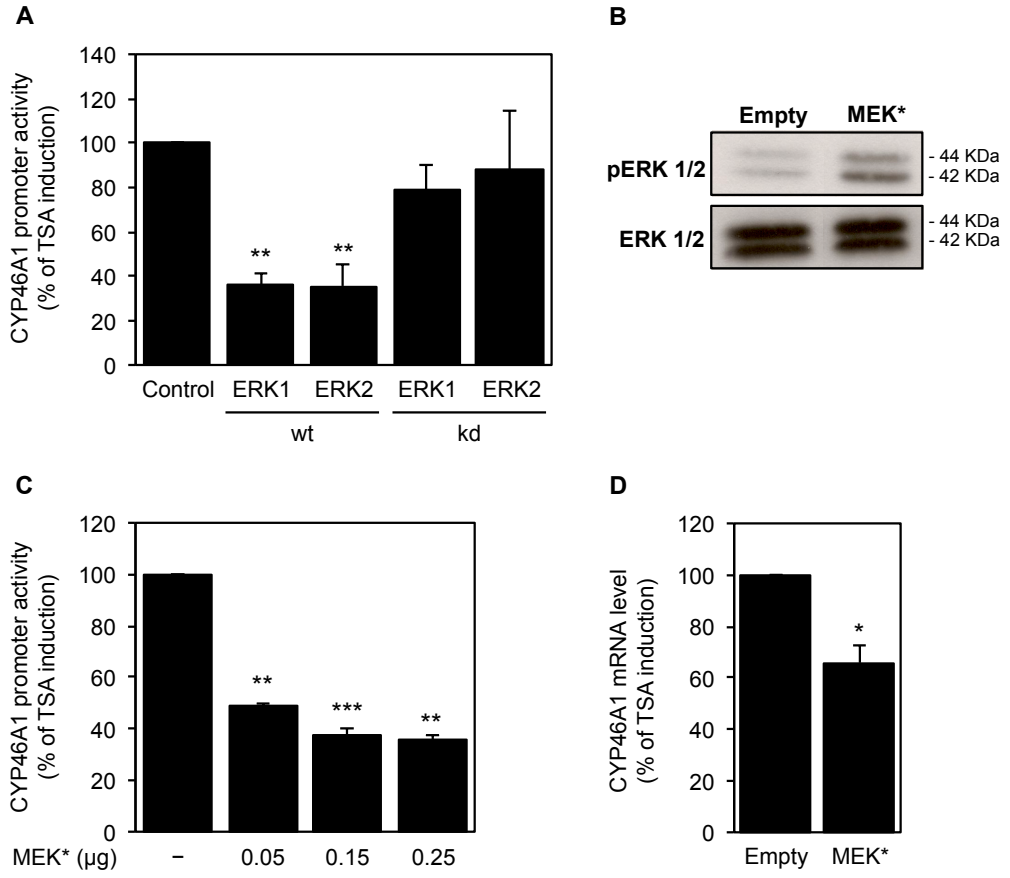

Figure 3.2. Overexpression of the MEK-ERK kinases partially impairs TSA-mediated increase in *CYP46A1* expression. A) The 0.12pGL2 plasmid was transfected with ERK1 and ERK2 *wild type* and mutated forms into SH-SY5Y cells. Twenty-four hours after transfection, cells were treated with or without 250 nM TSA for 16 h. Normalized luciferase activities were expressed as mean values ± SEM of duplicates for a minimum of three independent experiments. B) Western blot analysis of p-ERK1/2 and ERK1/2 protein level in SH-SY5Y cells after overexpression of a constitutively active MEK expression plasmid (MEK*). C) The 0.12pGL2 plasmid was transfected with different amounts of MEK* plasmid into SH-SY5Y cells. Twenty-four after transfection, cells were treated with or without 250 nM TSA for 16 h. Normalized luciferase activities were expressed as mean values ± SEM of duplicates for a minimum of three independent experiments. D) Real-time PCR analysis of CYP46A1 mRNA transcript levels in SH-SY5Y cells overexpressing the MEK* plasmid and treated with or without 250 nM TSA for 16 h. Values were normalized to the internal standard β-actin and are expressed as percentage of induction relative to TSA-treated cells. Data represent mean values ± SEM of at least three independent experiments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The transactivation studies revealed that the TSA induction of the 0.12pGL2 promoter construct activity was significantly diminished in the presence of the *wild type* forms of both ERK1 and ERK2, whereas the mutated proteins did not have any effect on the promoter activity (Fig. 3.2A; ANOVA one-way test: $F = 11.69$, df = 4, $p < 0.01$; Tukey HSD: ERK1 wt $p < 0.01$, ERK2 wt $p < 0.01$). Since we detected a similar effect of the overexpression of both ERK1 and ERK2 in the *CYP46A1* induction by TSA, the reporter activity analysis was also performed after transfection of an expression plasmid coding for a constitutively active form of MEK1 (MEK1*), which is the upstream kinase of ERK1/2. Western-blot analysis confirmed the significant increase in ERK1/2 phosphorylation after MEK1* transfection (Fig. 3.2B). As expected, overexpression of MEK1 resulted in a significant decrease in the TSA induction of the 0.12pGL2 promoter construct activity (Fig. 3.2C; ANOVA one-way test: $F = 22.3$, df = 3, $p < 0.001$; Tukey HSD: 0.5 μg and 2 μg MEK* $p < 0.01$, 1 μg MEK* $p < 0.001$). Furthermore, transfection of MEK1 expression plasmid also inhibited the TSA effect on the CYP46A1 mRNA level. Indeed, by real-time PCR we observed a decrease of 30% in the TSA-mediated increase of CYP46A1 mRNA levels (Fig. 3.2D).

3.4.3. TSA treatment promotes ERK1/2 dephosphorylation that is prevented in the presence of okadaic acid

Since the MEK-ERK signaling pathway clearly participates in the TSA-mediated derepression of *CYP46A1* gene, it prompted us to investigate whether TSA treatment could affect ERK1/2 activity. Western-blot analysis of total protein extracts from SH-SY5Y cells treated with 250 nM TSA for different time points revealed that TSA decreases ERK1/2 phosphorylation as soon as 30 min after treatment, being the control levels restored 2 h after the insult (Fig. 3.3A). Moreover, the TSA effect on ERK1/2 activity was found to be dose-dependent, since treatment with increasing concentrations of TSA for 30 min induced a gradual dephosphorylation of these kinases. TSA specifically affects the MEK-ERK pathway, since western-blot analysis revealed that phosphorylation levels of JNK, another member of the MAPK family, remained unchanged after TSA treatment (Fig. 3.3A).

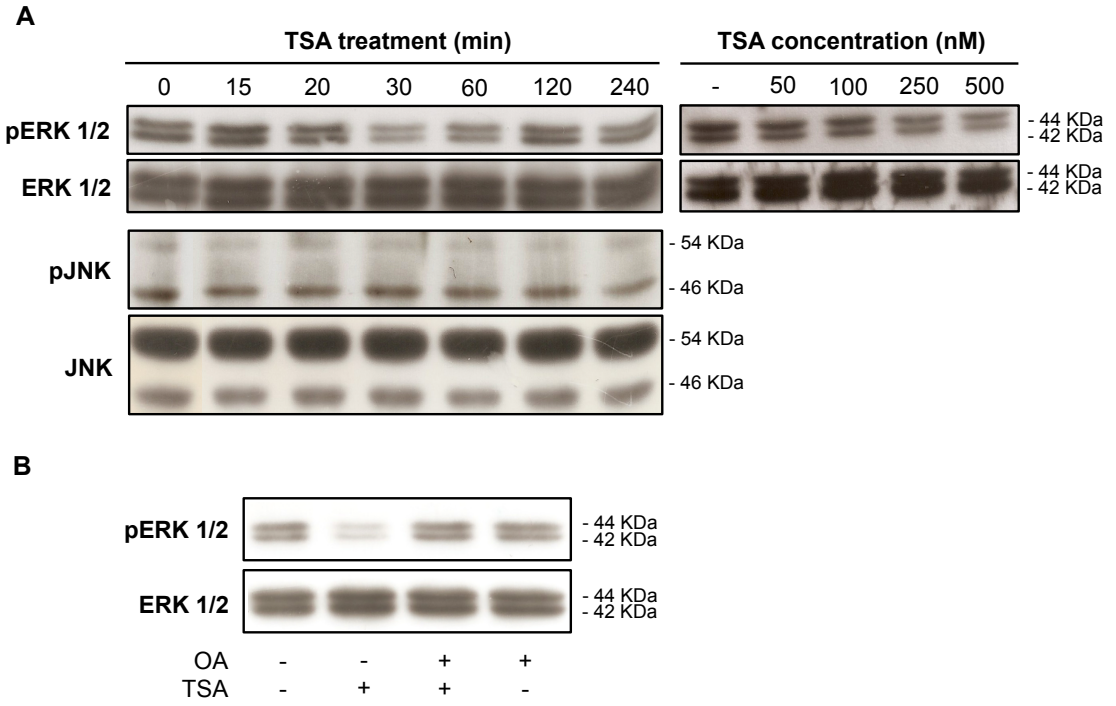

Figure 3.3. Okadaic acid prevents ERK1/2 dephosphorylation induced by TSA treatment. Western blot analysis of p-ERK1/2, ERK1/2, p-JNK, and JNK protein level in SH-SY5Y cells after treatment with 250 nM TSA for the indicated time points or with different concentrations of TSA for 30 min (A) and with 10 nM OA for 1 h, with or without 250 nM TSA, for 16 h (B). The results shown are representative of those obtained in three independent experiments.

Interestingly, pre-treatment of SH-SY5Y cells with OA completely reversed the decrease in ERK1/2 phosphorylation (Fig. 3.3B). In fact, inhibition of both PP1 and PP2A has been described to positively modulate ERK1/2 activity (Mao *et al.* 2005, Hedou *et al.* 2008), nevertheless we missed to identify the specific protein phosphatase inhibited by OA and involved in *CYP46A1* induction by TSA. On one hand the TSA effect was not affected by pre-treatment with tautomycin at the concentrations that specifically inhibit PP1, and on the other hand silencing of PP2A catalytic subunit by siRNA transfection had no effect on *CYP46A1* activation (data not shown).

Taken together these results suggest that early modulation of ERK1/2 activation by TSA can be critical for the end point effect on *CYP46A1* transcription. Since protein phosphatase inhibition by OA prevents ERK1/2 dephosphorylation, an OA-sensitive protein phosphate, different from PP1 and PP2A, seems essential for the modulation of ERK1/2 activity by TSA.

3.4.4. The impairment of TSA-mediated induction of *CYP46A1* expression by protein phosphatase inhibition is independent of histone hyperacetylation

Due to the drastic effect of OA pre-treatment on the TSA induction of CYP46A1 mRNA levels, we evaluated if the histone hyperacetylation of the promoter induced by TSA (Nunes *et al.* 2010) was being affected by OA administration. ChIP was performed with an antibody against tetra-acetylated histone H4 (K5, K8, K12 and K16) and chromatin isolated from SH-SY5Y cells treated with 250 nM TSA for 30 min (Fig. 3.4A). As expected an increase in H4 acetylation level was observed in the -1kb region of the *CYP46A1* promoter after TSA exposure (Nunes *et al.* 2010), however, pre-treatment with OA did not induce any significant change in the level of this histone marker (ANOVA one-way test: $F = 55.515$, df = 7, $p < 0.001$; Tukey HSD $p < 0.001$). Concomitantly, no differences in the recruitment of RNA pol II to the proximal promoter were observed between cells treated with 250 nM TSA for 30 min, in the presence or absence of OA, suggesting that, at least the initial transcription activation is not being affected (Fig. 3.4B; ANOVA one-way test: $F = 31.184$, df = 7, $p < 0.001$; Tukey HSD: TSA $p < 0.001$, OA and TSA $p < 0.05$). Indeed, a time-course analysis of CYP46A1 mRNA accumulation after TSA treatment, with or without OA, by real-time PCR corroborated this result (Fig. 3.5). The repressive effect of OA on *CYP46A1* activation by TSA was only evident from 6 h treatment onward. The OA repression of *CYP46A1* activation by TSA is therefore independent of the early and transient histone hyperacetylation at the promoter region, demonstrating that later events, other than chromatin remodeling, are also crucial for the HDACi effect.

3.4.5. TSA treatment triggers the release of p-ERK1/2 from *CYP46A1* promoter in a time-dependent manner

The presence of MAPKs on gene promoters, as part of the transcriptional complexes, has already been suggested, although ERK1/2, in contrast to ERK5 that contains a transcriptional activation domain (Kasler *et al.* 2000), is unable to directly bind DNA.

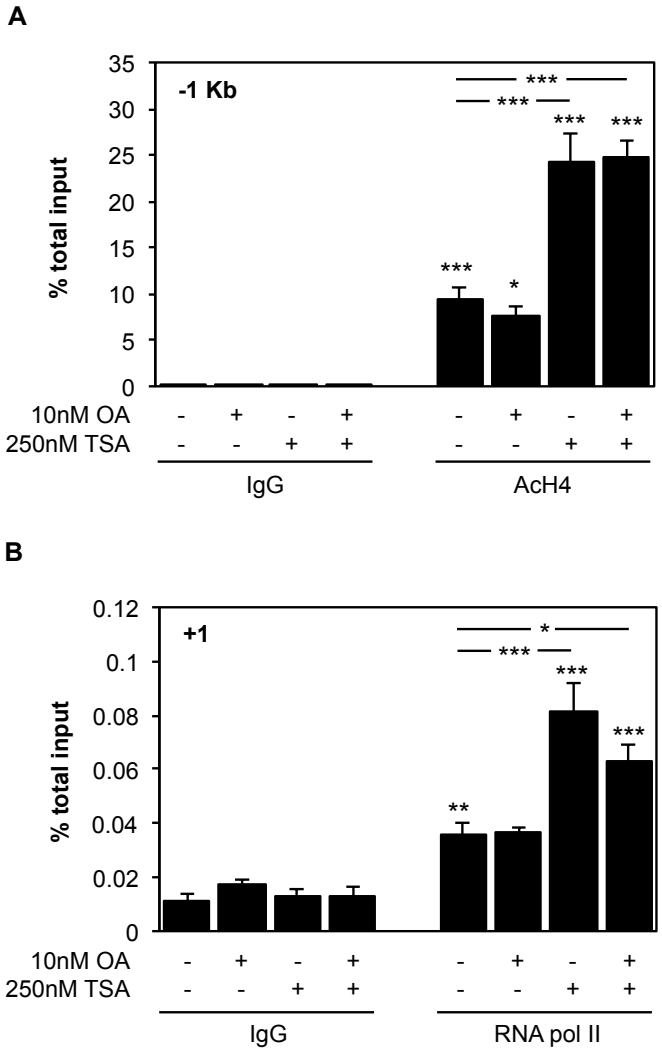

Figure 3.4. Histone hyperacetylation induced by TSA at *CYP46A1* promoter is not affected by okadaic acid pre-treatment. ChIP was performed using chromatin prepared from SH-SY5Y control cells and cells pretreated 1 h with 10 nM OA and treated for 30 min with or without 250 nM TSA. After chromatin precipitation with an anti-AcH4 (A) and –RNA pol II (B) antibodies, the recovered DNA was evaluated by real-time PCR. Results represent means ± SEM of at least three independent experiments and are expressed as percentage of total input (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

To investigate whether the effect of TSA on ERK1/2 can affect binding/activity of transcription factors to the *CYP46A1* promoter, we considered the idea that ERK1/2 dephosphorylation may induce its dissociation from chromatin-bound transcription factor complexes. To test this idea, we evaluated the recruitment of this kinase to the *CYP46A1* proximal promoter by ChIP (Fig. 3.6).

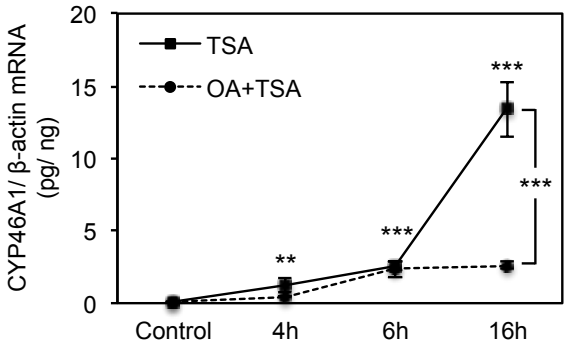

Figure 3.5. Okadaic acid inhibits the TSA-mediated activation of *CYP46A1* gene in a time-dependent manner. Real-time PCR analysis of CYP46A1 mRNA transcript levels in SH-SY5Y cells treated with 250 nM TSA for different time points pretreated or not 1 h with 10 nM OA. Values were normalized to the internal standard β-actin and are expressed as pg of CYP46A1 mRNA per ng of β-actin mRNA. Data represent means ± SEM of at least three independent experiments (** $p < 0.01$; *** $p < 0.001$).

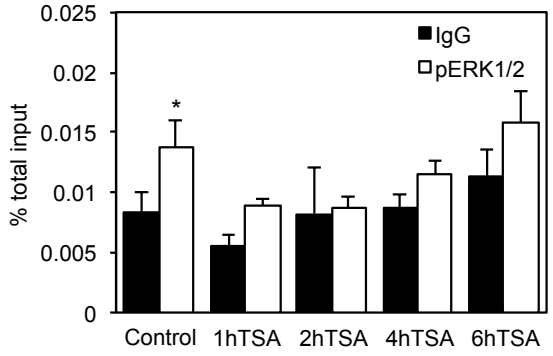

Figure 3.6. TSA treatment promotes p-ERK1/2 detachment from the *CYP46A1* promoter. ChIP was performed using chromatin prepared from SH-SY5Y control cells and cells treated with 250 nM TSA for the indicated time points. After chromatin precipitation with an anti-p-ERK1/2 antibody, the recovered DNA was evaluated by real-time PCR. Results represent means ± SEM of at least three independent experiments and are expressed as a percentage of total input (* $p < 0.05$).

The analysis by real-time PCR of the DNA fragments recovered after immunoprecipitation with an antibody against the phosphorylated form of ERK1/2 (p-ERK1/2) revealed the presence of this kinase in the proximal promoter of *CYP46A1*, in untreated cells. Concomitantly with the decrease in the total levels of p-ERK1/2, only 1h after TSA treatment we could no longer detect association of ERK1/2 to the promoter. Interestingly, ERK1/2 remained dissociated from the *CYP46A1* promoter even after 6 h of TSA treatment. Surprisingly, in contrast to what we previously observed for total protein

levels, the pre-treatment with OA does not impair detachment of p-ERK1/2 from the promoter in the first 6 h of treatment (data not shown).

Overall these results suggest that the transient reduction in ERK1/2 activity, 30 min after TSA treatment, induces a release of p-ERK1/2 from *CYP46A1* promoter that lasts at least for 6h, and is most likely required for the increase of CYP46A1 mRNA. Although OA is preventing TSA-dependent ERK1/2 dephosphorylation very rapidly, as previous assessed by immunodetection in whole cell lysates (Fig. 3.3), the impairment of TSA-mediated activation of *CYP46A1* transcription is probably due to an accumulation of p-ERK1/2 at the promoter at later time points, that gradually counteracts the TSA effect.

3.4.6. TSA affects Sp3 activity at the *CYP46A1* promoter level in a MEK-ERK1/2 dependent manner

In our previous work regarding the effect of histone deacetylase inhibition on *CYP46A1* transcription (Nunes *et al.* 2010), we described that Sp3 binding activity to specific Sp-responsive elements of the *CYP46A1* promoter was decreased in nuclear extracts from SH-SY5Y treated with TSA from 4 h onward, attaining control levels 24 h after TSA treatment. Due to the striking effect of OA on TSA activation of *CYP46A1* expression 16 h after HDAC inhibition (Fig. 3.5), we decided to evaluate if pre-treatment with OA was affecting Sp3 binding activity at this time point. We performed an EMSA with a probe encompassing one of the Sp-binding sites on the *CYP46A1* proximal promoter, the Sp-RE-B probe, and nuclear extracts from SH-SY5Y cells treated with 250 nM TSA for 16 h, in the presence or absence of OA (Fig. 3.7A). At this time point of TSA treatment, a decrease in Sp3 binding activity was still evident, and the inhibition of PPs with OA completely reversed the TSA effect, suggesting that Sp3 binding activity is being regulated by transient phosphorylation/dephosphorylation. To evaluate if OA was also affecting the binding/recruitment of Sp3 to *CYP46A1* proximal promoter we performed ChIP assays (Fig. 3.7B). No significant changes in Sp3 binding were observed after 16 h treatment with TSA, OA or both. We had previously observed a discrepancy between the results obtained with gel-shift regarding differences in Sp3 binding activity, and the recruitment of this transcription factor to the promoter determined by ChIP (Nunes *et al.* 2010).

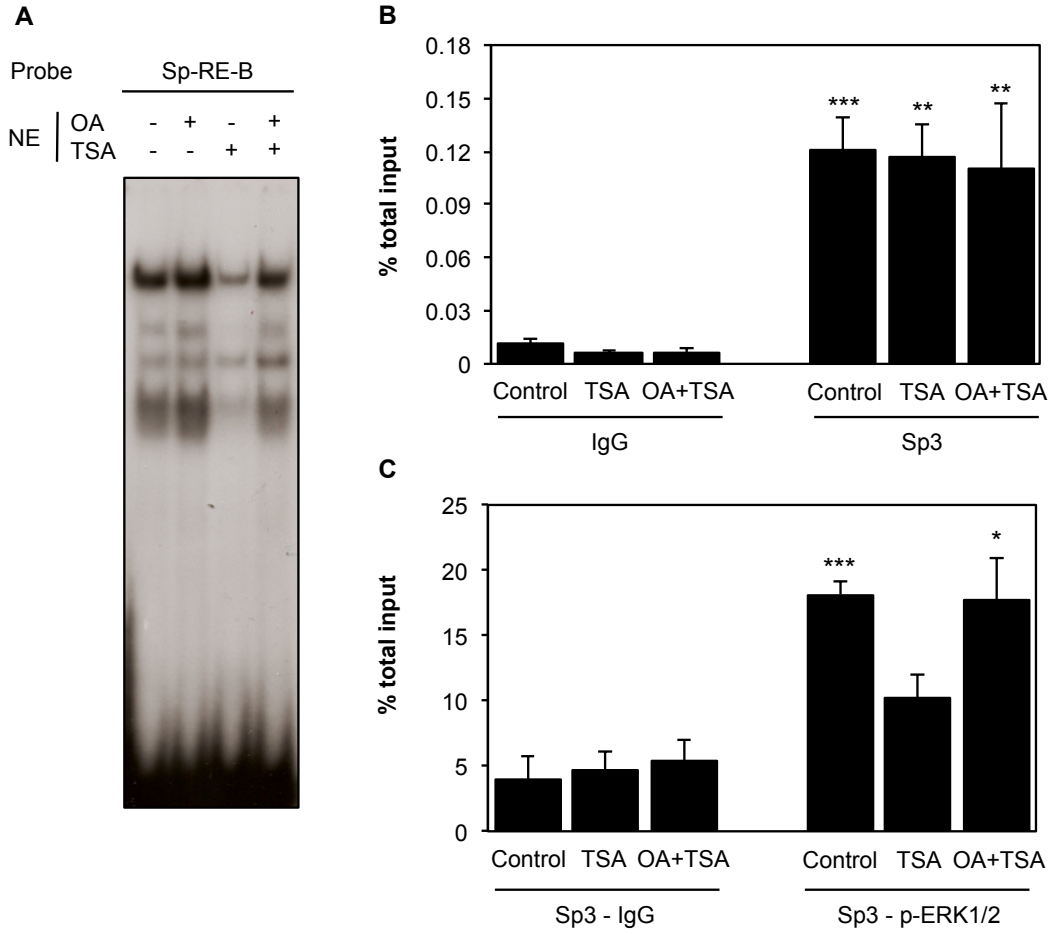

Figure 3.7. Sp3 binding activity is affected by okadaic acid treatment. EMSA (A), ChIP (B), and re-ChIP (C) were performed using nuclear extracts and chromatin, respectively, prepared from SH-SY5Y cells pretreated 1 h with 10 nM OA or treated for 16 h with or without 250 nM TSA. For the EMSA, a double-stranded oligonucleotide corresponding to the Sp-RE-B site was used as a probe. In the ChIP and re-ChIP assay, after chromatin precipitation with an anti-Sp3 and -p-ERK1/2 antibodies, the recovered DNA was evaluated by real-time PCR. Results represent means ± SEM of at least three independent experiments and are expressed as percentage of total input (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

These differences are most likely due to the distance between the several Sp-RE sites in the GC-rich proximal promoter (ten putative Sp-binding sites in the region encompassed by nucleotides -417 to -64), which is smaller than average chromatin fragments produced during sonication (500-900 bp), and therefore makes it impossible to discriminate between binding of Sp proteins to the different binding sites. Since the MEK-ERK pathway is mediating the TSA activation of *CYP46A1* gene and PPs inhibition is affecting Sp3 binding activity we hypothesized that ERK1/2 was modulating Sp3 activity. To assess if ERK1/2 is affecting Sp3 phosphorylation at the chromatin level, we performed re-ChIP analysis, and evaluated the association of Sp3 and p-ERK1/2 to

the same chromatin fragments of the *CYP46A1* promoter (Fig. 3.7C). In control cells we observed that both Sp3 and p-ERK1/2 share the same promoter fragments, whereas 16 h after TSA treatment we can no longer detect the association of this two proteins to the same regions. Pre-treatment of SH-SY5Y cells with OA completely restored the co-occupancy of Sp3-p-ERK1/2 in the *CYP46A1* promoter fragments.

Overall, these results demonstrate that TSA affects Sp3 activity in a MEK-ERK dependent manner, which is an essential event for the HDACi-mediated derepression of *CYP46A1* gene. In the presence of OA, an accumulation of p-ERK1/2 in the Sp3-containg fragments of *CYP46A1* promoter occurs, leading to phosphorylation-dependent modulation of Sp3 activity, and consequently a decrease in the TSA induction of *CYP46A1* expression.

3.4.7. Okadaic acid inhibits *CYP46A1* basal expression in NT2N human *post-mitotic* neurons

Since changes in the activity of protein phosphatases (Gong *et al.* 1995, Qian *et al.*), and in the levels of 24(S)-hydroxycholesterol (Lutjohann *et al.* 2000, Papassotiropoulos *et al.* 2002, Zuliani *et al.* 2011) have been described in AD patients, we were also interested in investigating whether these pathways could act directly as modulators of *CYP46A1* transcription. We started by evaluating *CYP46A1* promoter activity after treatment with kinase and phosphatase inhibitors (Fig 3.8A). The 0.12pGL2 promoter reporter construct was transfected in SH-SY5Y cells and 24 h after cells were treated for 16 h with the kinase inhibitors H89, PD98059, SP600125 or with the protein phosphatase inhibitor OA. As previously observed for the TSA effect, specific inhibition of the MEK-ERK pathway significantly increased *CYP46A1* promoter activity (ANOVA one-way test: $F = 4.255$, df = 3, $p < 0.05$; Tukey HSD $p < 0.05$) while OA treatment had the opposite effect, resulting in a decrease in the activity to approximately 50% of the control levels (ANOVA one-way test: $F = 6.647$, df = 3, $p < 0.01$; Tukey HSD $p < 0.01$). Accordingly, real-time PCR analysis revealed that MEK-ERK inhibition, by treatment with U0126 and PD98059 for 16 h, induces a 2.5-fold increase in SH-SY5Y CYP46A1 mRNA level (ANOVA one-way test: $F = 10.403$, df = 2, $p < 0.001$; Tukey HSD: U0126 $p < 0.01$, PD98059 $p < 0.05$) (Fig. 3.8B).

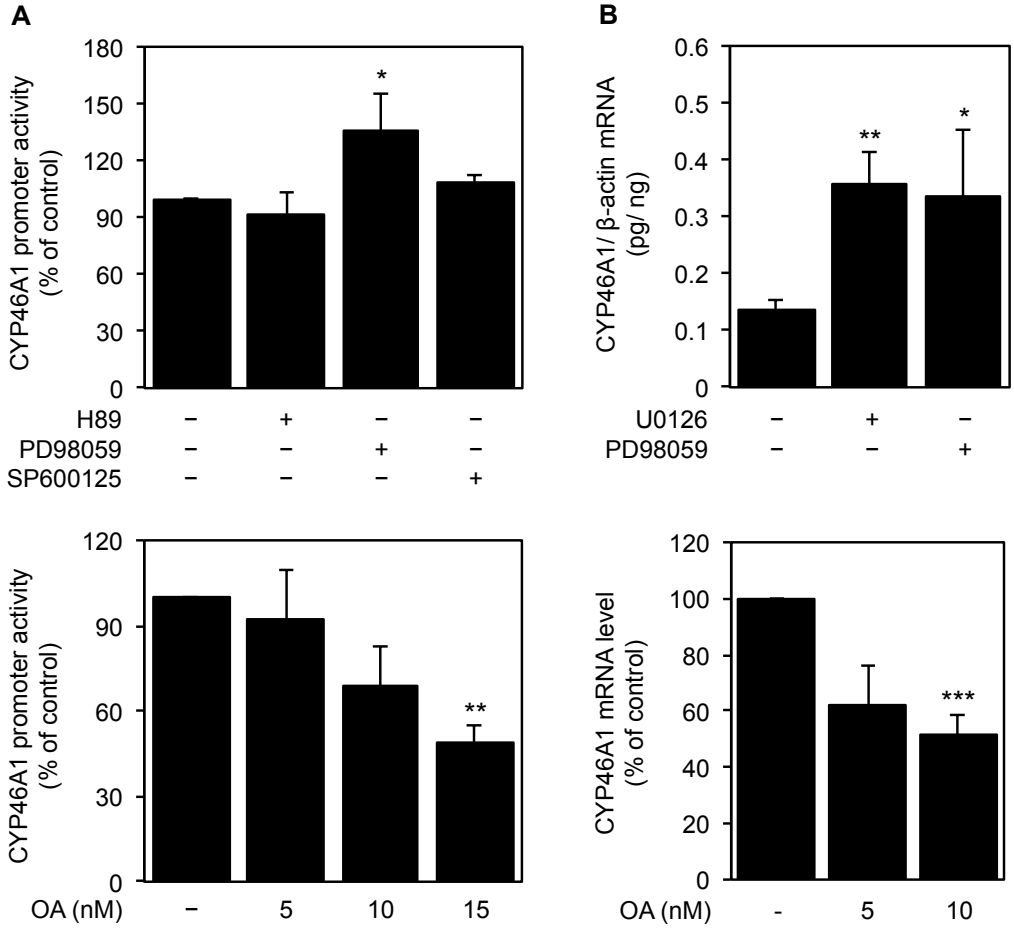

Figure 3.8. Effect of kinase/phosphatase chemical inhibitors on *CYP46A1* basal expression. A) The 0.12pGL2 plasmid was transfected into SH-SY5Y cells. Twenty-four hours after transfection, cells were incubated with 5 μM H89, 10 μM PD98059, 10 μM SP600125, or with the indicated concentrations of OA for 16 h. Normalized luciferase activities were expressed as mean values ± SEM of duplicates for a minimum of three independent experiments. B) Real-time PCR analysis of CYP46A1 steady-state mRNA transcripts level in SH-SY5Y and NT2N cells treated, respectively, with 10 μM U0126 or 10 μM PD98059 and with the indicated doses of OA for 16 h. Values were normalized to the internal standard β-actin. Data represent means ± SEM of at least three independent experiments and are expressed as percentage of induction relative to vehicle-treated cells (NT2N) or as pg of CYP46A1 per ng of β-actin (SH-SY5Y) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Since SH-SY5Y cells express very low levels of CYP46A1 mRNA, making it impossible to determine differences in gene expression that result in transcription inhibition, we treated human NT2 *post-mitotic* neurons (NT2N), with different concentrations of OA for 16 h, to evaluate if protein phosphatase inhibition also affects the basal transcription of this gene (Fig. 3.8B). Indeed, we have previously shown that NT2N cells are a suitable model to study both *CYP46A1* transcription and neuronal cholesterol metabolism, since these cells express high levels of CYP46A1 mRNA and

protein, and increased production of 24OHC (Milagre *et al.* 2012a, Milagre *et al.* 2012b). Real-time PCR analysis revealed that OA (10 nM) is able to inhibit the basal expression of *CYP46A1* gene resulting in a decrease of approximately 50% in the mRNA transcripts level (ANOVA one-way test: $F = 18.974$, df = 2, $p < 0.001$; Tukey HSD $p < 0.001$). These results point out that both serine-threonine protein phosphatases and the MEK-ERK signaling pathway are involved in the regulation of *CYP46A1* basal transcription, apart from their pivotal role in the derepression by histone deacetylase inhibition.

3.5. Discussion

Understanding *CYP46A1* transcriptional regulation required a new perspective because of the beneficial effects arising from overexpressing this enzyme in a mouse model of AD (Hudry *et al.* 2010). We and others have shown that HDACi are one of the few classes of compounds known to induce *CYP46A1* expression (Nunes *et al.* 2010, Shafaati *et al.* 2009). In our previous study we showed that the histone deacetylase inhibitor TSA induced a transient histone hyperacetylation of *CYP46A1* promoter, which significantly increased *CYP46A1* transcription in SH-SY5Y neuroblastoma cells (Nunes *et al.* 2010). In the same work a specific region in the proximal promoter that encompasses four Sp-binding sites was identified as essential for the TSA activation of *CYP46A1* gene. Binding of both Sp1 and Sp4 transcription factors was found to be indispensable for the TSA effect. On the other hand, a decrease in Sp3 binding activity was observed after TSA treatment.

The fact that histone deacetylation was observed at a time point of TSA treatment where the HDAC/HAT equilibrium should still be favoring acetylation lead us to investigate which mechanism(s), other than histone hyperacetylation, were involved in the TSA-mediated increase in *CYP46A1* gene expression. The studies presented here clearly identify the MEK-ERK signaling pathway as critical for the modulation of Sp3 transcriptional activity, and the activation of *CYP46A1* by TSA. The Sp family of transcription factors, including Sp3, is highly regulated at the post-translational level. Although Sp3 is able to both up- (Pages 2007, Bergeron *et al.*) and down-regulate gene transcription (Petrovic *et al.* 2009, Tsika *et al.* 2004, Seyed & Dimario 2008), phosphorylation is generally associated with increased DNA binding activity and,

consequently, increased transcriptional activation (Pages 2007, Bakovic *et al.* 2003). Modifications such as acetylation have also been described to both activate (Ammanamanchi *et al.* 2003) and repress (Braun *et al.* 2001) Sp3 transcriptional activity, whereas sumoylated Sp3 significantly represses transcription (Sapetschnig *et al.* 2002, Ross *et al.* 2002, Stielow *et al.* 2008).

The participation of the MEK-ERK signaling pathway in the derepression of *CYP46A1* gene by TSA was confirmed by pre-treatment of neuroblastoma cells with specific inhibitors of this pathway (PD98059 and U0126) and overexpression of the *wild type* and catalytic inactive forms of both ERK1 and 2, and a constitutively active form of MEK1. From these experiments we confirmed that inhibition of the MEK-ERK pathway resulted in the potentiation of the TSA effect. In contrast, inhibition of protein phosphatase activity impaired the TSA-mediated activation of *CYP46A1* gene, pointing to a dephosphorylation event as indispensable for the HDACi effect. TSA and other HDACi have been associated with increased kinase activity; namely, sodium butyrate induces erythroid differentiation of K562 cells by inhibition of ERK and activation of p38 signal transduction pathways (Witt *et al.* 2000), and stimulates PKC activation in the same cellular model (Rivero & Adunyah 1998). However, TSA was also shown to significantly decrease phosphorylation of both Akt and ERK1/2 in a glioblastoma cell line (Chen *et al.* 2005). In our cellular model, the SH-SY5Y neuroblastoma cells, TSA induced an early marked but transient decrease in ERK1/2 phosphorylation, as soon as 30 min after TSA treatment, resulting in the dissociation of these kinases from the *CYP46A1* promoter for at least 6 h. Since this effect perfectly correlated with the decreased binding activity of Sp3 protein in the presence of TSA, and due to the established importance of the Sp family of transcription factors in the HDACi derepression of *CYP46A1* gene, we hypothesized TSA was modulating Sp3 transcriptional activity in a ERK1/2 dependent manner. In fact, Sp3 has already been described as a target of the ERK1/2 MAPK family (Bakovic *et al.* 2003, Pages 2007).

Interestingly, OA pre-treatment completely reversed the decrease in Sp3 binding activity induced by TSA, strengthening our hypothesis. Although PP1 has been demonstrated to dephosphorylate Sp3 (Chu *et al.* 2003), it seems that the protein phosphatase involved in the process is not directly modulating Sp3 phosphorylation, but instead ERK1/2 activity, since treatment with OA efficiently reverted the ERK1/2 dephosphorylation observed after TSA treatment. The concentration of the protein

phosphatase inhibitor used in this study (10 nM OA) should specifically inhibit PP2A, and not PP1. However silencing of PP2A catalytic subunit alpha (PP2Ac) did not have any effect on *CYP46A1* activation by TSA, confirming that other OA-sensitive phosphatase is involved in the TSA effect. In fact, at this concentration OA can also be inhibiting other protein phosphatases such as PP4 or PP5, which can only be discriminated by specific siRNA silencing. We have verified that most studies using OA implicate PP1 or PP2A in biological processes and neglect the possibility that some of the biological effects of OA are due to the inhibition of PP4 or PP5. Our results highlight the importance of the identification of the protein phosphatase involved in the control of ERK1/2 activity in neuronal cells.

The fact that alterations in the phosphorylation level of ERK1/2 correlated with CYP46A1 mRNA level and Sp3 binding activity point to a relationship between Sp3 phosphorylation/dephosphorylation and the TSA effect. Importantly, impairment of TSA-mediated activation of *CYP46A1* gene by inhibition of protein phosphatases activity resulted in the blockage of ERK1/2 dephosphorylation, without affecting histone hyperacetylation of the promoter or the initial recruitment of RNA pol II. The repressive effect of protein phosphatase inhibition, by pre-treatment with OA, led to an increase in the association of Sp3 with activated ERK1/2 at the *CYP46A1* proximal promoter, which most likely induces Sp3 phosphorylation, and anchoring of co-repressors complexes to the promoter at later time points. The Sp3 role in this context is distinct from what we have previously observed after treatment of neuroblastoma cells with the demethylating agent 5-aza-2-deoxicytidine (Milagre *et al.* 2010). In that work a decrease in Sp3 protein level was observed after drug treatment concomitantly with a detachment of both Sp3 and HDACs from *CYP46A1* proximal promoter, demonstrating that this transcription factor is modulating transcription by recruitment/detachment of chromatin remodeling agents. An interesting hypothesis is that Sp3 phosphorylation by ERK1/2 is favoring other post-translational modifications, such as acetylation or sumoylation, which can also be responsible for Sp3-mediated repression. Indeed, it has been described that sumoylation and ubiquitination of Sp1 transcription factor can be mediated by phosphorylation (Spengler *et al.* 2008), supporting a novel mechanism of Sp-dependent gene regulation. This is also a feature of the ERK1/2 MAPK family to control transcriptional regulation, as players between phosphorylation-dependent post-translational modifications that can

affect protein's transcriptional activity, from ubiquitination to sumoylation and acetylation (Whitmarsh 2007).

We have recently described the differentiation of NT2 cells into *post-mitotic* neurons (NT2N) as a suitable cellular model to study *CYP46A1* transcriptional regulation (Milagre *et al.* 2012a). Treatment of NT2N cells with 10 nM OA for 16 h significantly reduced CYP46A1 mRNA level. This effect is particularly relevant in the context of AD. This neurological condition has been widely linked to compromised protein phosphatase activity, which is responsible for one of the hallmarks of the disease, tau protein hyperphosphorylation. Indeed, our results suggest that the decreased PP2A and PP5 activity described in the brains of AD patients (Liu *et al.* 2005, Liu & Wang 2009, Gong *et al.* 1995) can also have an adverse consequence in brain cholesterol metabolism. Interestingly, changes in the flux of 24OHC, from the brain into the circulation have been reported in patients with AD, with reduced circulating levels of this oxysterol in the more advance cases of neurodenegeration (Papassotiropoulos *et al.* 2000).

Taken together these results demonstrate the critical participation of the MEK-ERK transduction pathway in the HDACi-mediated activation of *CYP46A1* gene, in a mechanism that is independent of histone hyperacetylation of the promoter. TSA treatment induces the dissociation of p-ERK1/2 from the promoter, in a PP1/PP2A independent manner, and specifically from the Sp3-containing DNA fragments, which leads to a decrease in Sp3 repressor transcriptional activity and probably the recruitment of other co-repressor proteins to the *CYP46A1* promoter.

3.6. Acknowledgments

We deeply thank Dr. Melanie Cobb (University of Texas, Southwestern Medical Center, Dallas, TX, USA) for kindly providing the expression vectors for the *wild type* and catalytic inactive forms of ERK1 and 2 (pCEP4-ERK1-wt and K71R; 3xflag-pCMV7-ERK2-wt and KR) and to Dr. Roger J. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA, USA) for the plasmid encoding a constitutively active form of MEK1 (pCMV-deltaN3-MEK1*).

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CHAPTER 4

HISTONE DEACETYLASE INHIBITION DECREASES CHOLESTEROL LEVELS IN NEURONAL CELLS BY MODULATING KEY GENES IN CHOLESTEROL SYNTHESIS, UPTAKE AND EFFLUX

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(Manuscript submitted)

4.1. Abstract

Cholesterol is an essential component of the central nervous system and increasing evidence suggests an association between brain cholesterol metabolism dysfunction and the onset of neurodegenerative disorders. Interestingly, histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA) are emerging as promising therapeutic approaches in neurodegenerative diseases, but their effect on brain cholesterol metabolism is poorly understood. We have previously demonstrated that HDACi up-regulate *CYP46A1* gene transcription, a key enzyme in neuronal cholesterol homeostasis. In this study, we show that TSA modulates the transcription of other genes involved in cholesterol metabolism in human neuroblastoma cells, namely by up-regulating genes that control cholesterol efflux and down-regulating genes involved in cholesterol synthesis and uptake, thus leading to an overall decrease in total cholesterol content. Furthermore, co-treatment with the amphipathic drug U18666A, that can mimic the intracellular cholesterol accumulation observed in cells of Niemann Pick C patients, revealed that TSA can ameliorate the phenotype induced by pathological cholesterol accumulation, by restoring the expression of key genes involved in cholesterol synthesis, uptake and efflux and promoting lysosomal cholesterol redistribution. These results clarify the role of TSA in the modulation of neuronal cholesterol metabolism at the transcriptional level, and emphasize the idea of HDAC inhibition as a promising therapeutic tool in neurodegenerative disorders with impaired cholesterol metabolism.

4.2. Introduction

Brain cholesterol is an essential component of neuronal cell membranes and myelin sheets and is involved in several neuronal cellular functions, such as synaptogenesis and synaptic plasticity (Dietschy & Turley 2004). Therefore, it is not surprising that increasing evidence relates dysfunction in cholesterol metabolism to the aetiology of many neurodegenerative disorders. For instance, the major risk factor for Alzheimer's disease (AD) is the presence of the E4 isoform of apolipoprotein E, the major cholesterol transporter in the brain (Strittmatter & Roses 1996), while in Niemann-Pick type C (NPC) disease, mutations in the NPC1 and 2 proteins that affect intracellular cholesterol trafficking, are responsible for the pathology (Rosenbaum & Maxfield 2011). Moreover, cholesterol levels influence amyloid precursor protein processing; high cholesterol levels shift amyloid precursor protein processing towards production of the amyloid-β peptide, which in turn accumulates in neuritic plaques in AD (Vetrivel & Thinakaran 2010).

Due to the blood brain barrier, cholesterol metabolism in the central nervous system (CNS) is distinct from that in other tissues. In fact, the brain is unable to take-up cholesterol from circulation and relies completely on *de novo* synthesis (Zlokovic 2008). In the developing CNS, cholesterol synthesis is relatively high, but declines to low levels in the adult (Lutjohann *et al.* 1996), mainly due to a highly efficient recycling of brain cholesterol. Despite the efficiency of the cholesterol recycling machinery, the rate of cholesterol synthesis in the adult brain is larger than the rate of accumulation. Therefore, the brain relies on the conversion of cholesterol into 24(S)-hydroxycholesterol (24OHC) as the major mechanism of cholesterol elimination (Lutjohann *et al.* 1996, Bjorkhem *et al.* 1998, Xie *et al.* 2003). The enzyme responsible for 24(S)-hydroxylation of cholesterol is a cytochrome P450, CYP46A1, almost exclusively expressed in neurons (Lund *et al.* 1999). Interestingly, inactivation of Cyp46a1 was associated with a selective reduction of cholesterol synthesis (Lund *et al.* 2003), while a significant increase in several cholesterol precursors was observed in the brain of Cyp46a1 transgenic mice (Shafaati *et al.* 2011b). This suggests a close relation between synthesis and catabolism of cholesterol in the CNS.

The human CYP46A1 5'- flanking region has been characterized (Ohyama *et al.* 2006, Milagre *et al.* 2008). Unlike other P450 genes, CYP46A1 expression is not

dependent on its substrate level, and the promoter is unresponsive to a large number of ligands for different nuclear receptors (Ohyama *et al.* 2006). Nevertheless, we have demonstrated that CYP46A1 is significantly up-regulated during differentiation of human neuronal cells (Milagre *et al.* 2012a, Milagre *et al.* 2012b), and that chromatin-modifying agents, 5'-Aza-2'-deoxycytidine and trichostatin A (TSA), dramatically increase CYP46A1 transcription (Milagre *et al.* 2010, Nunes *et al.* 2010). These latter results suggest that histone deacetylase (HDAC) inhibitors (HDACi) can eventually be used to modulate brain cholesterol metabolism.

HDACs play a key role in histone acetylation homeostasis and in the regulation of fundamental cellular activities, such as transcription. A wide range of brain disorders is associated with imbalanced protein acetylation and treatment with HDACi has been shown to correct these deficiencies and has emerged as a promising new strategy for therapeutic intervention in neurodegenerative diseases. Namely, HDACi have been shown to have neuroprotective, neurotrophic and anti-inflammatory properties, while improving neurological performance and learning/memory in several disease animal models of Huntington's disease (Ferrante *et al.* 2003, Hockly *et al.* 2003, Gardian *et al.* 2005), spinal muscular atrophy (Chang *et al.* 2001, Avila *et al.* 2007), amyotrophic lateral sclerosis (Corcoran *et al.* 2004, Ryu *et al.* 2005, Petri *et al.* 2006), and experimental autoimmune encephalomyelitis (Camelo *et al.* 2005). Nevertheless, there is hardly any information about how pharmacological intervention in this pathway affects brain cholesterol metabolism. Only recently have HDACi been shown to correct cholesterol storage defects in human NPC1 mutant fibroblasts (Pipalia *et al.* 2011).

Herein, we show that treatment of SH-SY5Y neuroblatoma cells with the pan-HDACi TSA decreases cholesterol levels by inducing an increase in the expression of genes involved in cholesterol efflux and catabolism and a decrease in the transcription of cholesterologenic genes. Moreover, by treating cells with the chemical compound U18666A, which can mimic the accumulation of cholesterol in late-endosomal/lysosomal compartments observed in cells from NPC patients (Cenedella 2009), we have confirmed that TSA can ameliorate the phenotype induced by pathological cholesterol accumulation, by restoring the expression of key genes in cholesterol synthesis, uptake and efflux.

4.3. Methods

4.3.1. Reagents and antibodies

The chemical inhibitors TSA and U18666A {3-b-[2-(diethylamino) ethoxy]androst-5-en-17-one} were from Sigma (Sigma Aldrich Inc., St Louis, MO, USA). Filpin III (Sigma-Aldrich), the primary antibodies anti-lysosome-associated membrane protein 2 (LAMP-2) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-acetyl-histone 4 (AcH4) (Millipore, Bedford, MA, USA), and the secondary antibodies anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488 (Molecular Probes®, Invitrogen, Carlsbad, CA, USA) were used in the immunostaining.

4.3.2. Cell culture

The SH-SY5Y human neuroblastoma cell line was maintained as previously described (Milagre *et al.* 2008). Briefly, cells were maintained in low glucose DMEM (Sigma-Aldrich), at 37°C in humidified 5% CO_2. The media was supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco®, Invitrogen).

4.3.3. Expression analysis

Total cell RNA was extracted using Trizol Reagent (Invitrogen) following manufacturer's instructions. Real-Time PCR (qPCR) analysis for 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) and synthase (HMGCS), mevalonate kinase (MVK), sterol regulatory element-binding protein (SREBP) 1c and 2, LDL receptor (LDLR), apolipoprotein E (APOE), Niemann-Pick disease, type C1 (NPC1) and ATP-binding cassette transporter 1 (ABCA1) was performed using SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) and specific primers (Table 4.1). CYP46A1 mRNA levels were determined as previously described (Nunes *et al.* 2010). Results presented were obtained from at least three individual experiments and each sample was assayed in triplicate. The mRNA levels were normalized to the level of β-actin and expressed as fold change from controls, using the ΔΔCt method. Statistical analysis was performed using ΔCt values.

Table 4.1. List of primers used for qPCR

Gene	Sequence (5' – 3') / source
CYP46A1	CYP46A1 HS00198510_M1 Ref: 4331182 (Taqman®, Applied Biosystems)
β – actin	ACTB HS99999_M1 Ref.: 4310881E-060521 (Taqman®, Applied Biosystems)
HMGCR	5' ATAGGAGGCTACAACGCCCAT 3' (fwd) 5' TTCTGTGCTGCATCCTGTCC 3'(rev)
HMGCS	5' GGCACAGCTGCTGTCTTCAAT 3' (fwd) 5' ACCAGGGCATACCGTCCAT 3' (rev)
MVK	5' CTCCGATACCATCAAGGG 3' (fwd) 5' GCTCACACTCCAGGGAGA 3' (rev)
LDLR	5' CAGATATCATCAACGAAGC 3' (fwd) 5' CCTCTCACACCAGTTCACTCC 3' (rev)
SREBP1c	5' GGAGGGGTAGGGCCAACGGCCT 3' (fwd) 5' CATGTCTTCGAAAGTGCAATCC 3' (rev)
SREBP2	5' CAGCTGCACATCACAGGGAA 3' (fwd) 5' GTACATCGGAACAGGCGGAT 3' (rev)
APOE	5' GACTGGCCAATCACAGGC 3' (fwd) 5' CTGTCTCCACCGCTTGCTC 3' (rev)
NPC1	5' ACACCTTCTCTCTCTTTGCGGG 3' (fwd) 5' GCTTGTTCCATCTTCAGCACCTC 3' (rev)
ABCA1	5' CCTGTTTCCGTTACCCGACTC 3' (fwd) 5' ACAGGCGAGCCACAATGG 3' (rev)
β – actin	5' CTGGAACGGTGAAGGTGACA 3' (fwd) 5' AAGGGACTTCCTGTAACAATCCA 3' (rev)

4.3.4. Immunocytochemistry

Immunocytochemistry experiments were performed as described previously (Castro-Caldas *et al.* 2009). Cellular cholesterol was stained using 25 μg/ml filipin III in phosphate-buffered saline for 2 h at room temperature. The AcH4 antibody was used for nuclear staining. Control experiments for non-specific binding were performed in parallel by omission of the primary antibody. Fluorescence visualization was performed in an AxioScope.A1 microscope (Zeiss, Germany) with an AxioCam HRm camera (Zeiss). Fluorescence and co-localization quantification was performed with the ImageJ 1.46 software.

4.3.5. Total cholesterol levels

Total cell cholesterol was quantified using the Amplex® Red cholesterol assay kit (Invitrogen) according to the manufacturer's instructions. Briefly, cells were

resuspended in 1x reaction buffer, placed on ice for 30 min and sonicated. Samples were then diluted in 1x reaction buffer, and 50 µl were used to quantify cholesterol. Samples were placed in a 96-well plate and the reaction was initiated by adding 50 µl of the Amplex® Red reagent/HRP/cholesterol oxidase/cholesterol esterase working solution to each well. The reactions were incubated for 30 min at 37ºC. Fluorescence measurements were performed in a spectrofluorometer Infinite® M200 (Tecan, Männedorf, Switzerland). Results presented were obtained from at least three individual experiments and each sample was assayed in triplicate. Cholesterol levels were normalized with total protein levels and expressed as ng of cholesterol per µg of protein.

4.3.6. Statistical analysis

Statistical analysis was performed using the Student's t-test, the Kruskal-Wallis test with Dunn's *post-hoc* test and the ANOVA one-way test with the Tukey HSD *post-hoc* test or the Tukey HSD for unequal N (Spjotvoll/Stoline test). The analysis was performed using STATISTICA (data analysis software system), version 9.1 StatSoft, Inc. (2010) or Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

4.4. Results

4.4.1. Expression pattern of genes involved in cholesterol synthesis, uptake and efflux after TSA treatment of SH-SY5Y neuronal cells

We have previously shown that inhibition of HDAC activity by sodium butyrate, valproic acid and TSA can significantly up-regulate CYP46A1 gene expression (Nunes *et al.* 2010), and therefore could eventually be used to modulate brain cholesterol metabolism. Thus, the goal of this study was to evaluate the effect of HDAC inhibition in the expression levels of other key genes in cholesterol metabolism, namely those involved in synthesis, uptake and efflux.

SH-SY5Y cells were cultured in the absence or presence of 250 nM TSA for 16 h. Total RNA was extracted and mRNA levels were quantified by qPCR. Our results show a significant modulation in the expression levels of genes coding for proteins of the

mevalonate pathway, namely HMGCS, HMGCR and MVK, which were decreased to about 70%, 75% and 30% of control values, respectively (Fig. 4.1). Moreover, TSA also induced a significant 50% down-regulation of the LDLR gene. Since cholesterol homeostasis is transcriptionally controlled by members of the SREBP family of transcription factors (Sato 2010), we also determined the expression of SREBP1c and SREBP2. Nevertheless, we did not detect any significant changes in the SREBPs mRNA levels. Interestingly, while TSA seems to decrease biosynthesis and uptake, it has the opposite effect on cholesterol efflux, since it induces a significant 2-fold increase in the mRNA levels of the ABCA1 gene.

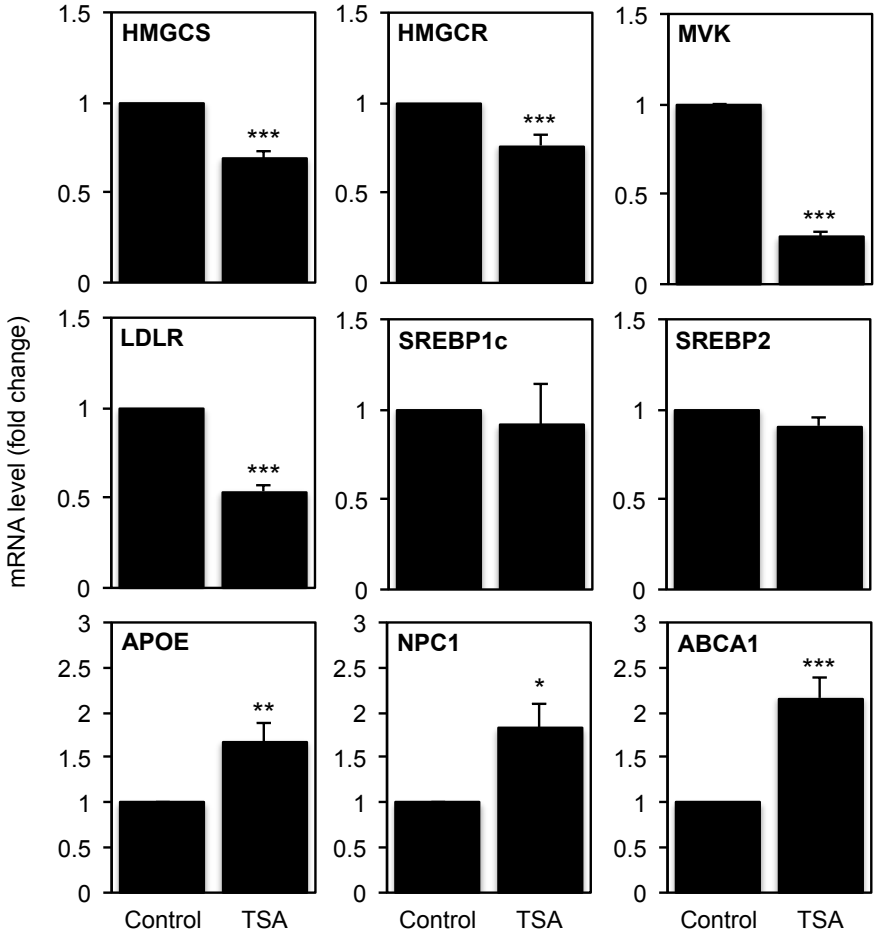

Figure 4.1. mRNA expression levels of genes involved in cholesterol synthesis, uptake, transport and efflux after TSA treatment. SH-SY5Y neuroblastoma cells were treated with 250 nM TSA for 16 h. mRNA transcript levels of HMGCS, HMGCR, MVK, LDLR, SREBP1c and SREBP2, APOE, NPC1 and ABCA1 were analyzed by real-time RT-PCR. Values were normalized to the internal standard β-actin and expressed as fold change relative to untreated cells. Values represent means ± SEM from at least three individual experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Moreover, a significant increase was also observed in the expression levels of the APOE (1.7-fold) and NPC1 (1.9-fold) genes, coding for proteins responsible for intercellular and intracellular cholesterol transport, respectively. In parallel, we have confirmed our previous results showing a significant increase in CYP46A1 mRNA after TSA treatment (data not shown).

These results suggest that TSA can modulate cholesterol homeostasis in neuroblastoma cells, since it up-regulates genes involved in cholesterol catabolism (CYP46A1) and efflux (ABCA1), and down-regulates genes involved in cholesterol synthesis (HMGCS, HMGCR and MVK) and uptake (LDLR).

4.4.2. TSA treatment decreases intracellular total cholesterol levels in SH-SY5Y neuronal cells

To determine if the observed changes in the expression levels of genes responsible for cholesterol homeostasis affect total cholesterol content, SH-SY5Y cells were cultured in the absence or presence of 250 nM or 500 nM TSA for 16, 24 and 48 h, and total cholesterol levels were determined with the Amplex Red cholesterol assay in whole-cell fractions (Fig. 4.2).

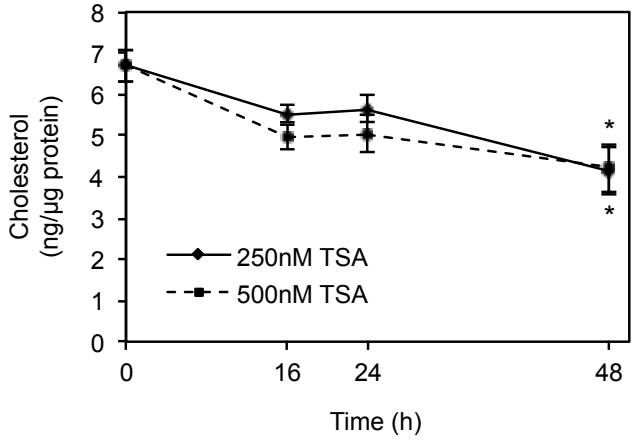

Figure 4.2. TSA treatment significantly decreases total cholesterol content in neuroblastoma cells. Quantification of total cholesterol was performed in SH-SY5Y neuroblastoma cells treated with 250 nM or 500 nM TSA for indicated time-points. Values were normalized to total protein content and expressed as ng of cholesterol per µg of total protein. Data represent means ± SEM from at least three individual experiments (* $p < 0.05$).

Our results showed a significant decrease of 40% in total cholesterol levels 48 h after TSA treatment (Kruskal-Wallis test, F = 18.30, df = 6, $p < 0.01$; Dunn, $p < 0.05$).

To further confirm that TSA could ameliorate the phenotype induced by pathological cholesterol accumulation, we pre-treated SH-SY5Y neuroblastoma cells with U18666A, a compound that can mimic the accumulation of unesterified cholesterol in late-endosomal/lysosomal compartments similar to that occurring in NPC1-deficient fibroblasts. The dynamics of intracellular cholesterol accumulation was visualized by fluorescence microscopy after filipin staining which can specifically label unesterified cholesterol. Moreover, in parallel we determined changes in total cholesterol levels, as above-mentioned. Untreated cells showed pale and diffuse filipin staining, whereas cells treated with U18666A revealed a characteristic intense and punctuate staining pattern (Fig 4.3A).

As expected, treatment with 3 μg/ml U18666A induced a significant increase in total cholesterol levels 72 h after treatment (8.66 ng ± 0.25 of cholesterol/μg of total cell protein vs. 11.86 ng ± 0.32 of cholesterol/μg of total cell protein) (ANOVA one-way test: $F = 28.57$, df = 3, $p < 0.001$; Tukey HSD, $p < 0.01$) (Fig. 4.3B). To confirm that TSA could ameliorate the NPC phenotype, we treated neuroblastoma cells with 3 μg/ml U18666A for 24 h, and then incubated cells for further 48 h in the presence or absence of 500 nM TSA. Interestingly, U18666A-induced cholesterol increase was abolished by TSA treatment (Tukey HSD, $p < 0.01$) (Fig. 4.3B).

4.4.3. TSA treatment partially reverts the effect of U18666A on cholesterol synthesis, uptake and efflux and promotes lysosomal cholesterol redistribution

It has been shown that cholesterol homeostasis in $NPC1^{-/-}$ mice was improved by treatment with valproic acid, a weak HDACi (Kim *et al.* 2007). More recently, HDAC inhibitors led to the correction of the NPC phenotype in cells with either one or two copies of the $NPC1^{I1061T}$ mutation, a correction mainly associated with increased expression of NPC1 protein (Pipalia *et al.* 2011). This is in accordance with our results that demonstrate the up-regulation of NPC1 gene after TSA treatment.

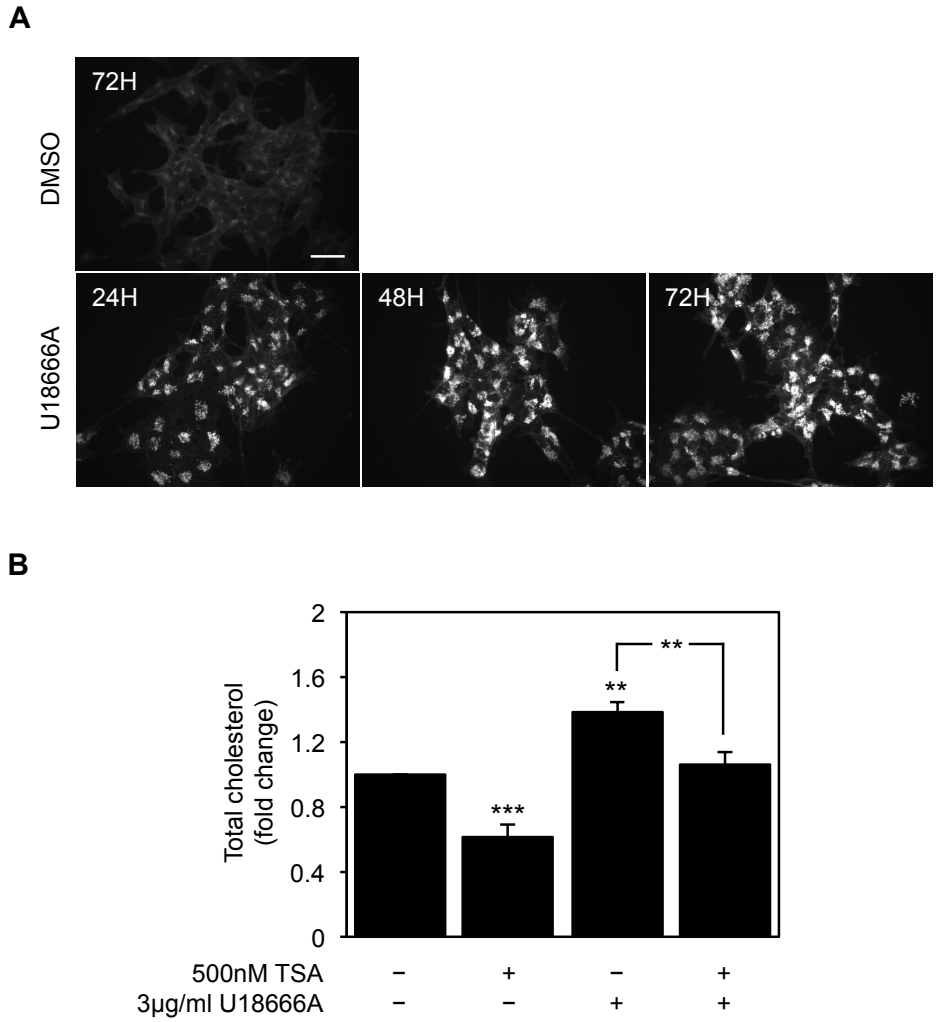

Figure 4.3. TSA reverts the increase in total cholesterol levels observed after U18666A treatment. A) Filipin III immunofluorescence staining of SH-SY5Y cells treated for the indicated time-points with 1 µg/ml U18666A or vehicle (*scale bar* = 40 µm). B) SH-SY5Y cells were treated with 3 µg/ml U18666A for 24 h and with or without 250 nM TSA for 48 h, and total cholesterol levels were determined. Values were normalized to total protein content and expressed as ng of cholesterol per µg of total protein. Data represent means ± SEM from at least three individual experiments (** $p < 0.01$, *** $p < 0.001$).

Moreover, since we observed that TSA restores cellular cholesterol content after U18666A treatment, we then evaluated how the expression of genes involved in cholesterol homeostasis was being affected by U18666A treatment, in the presence or absence of TSA. We analyzed the expression levels of five genes, involved in cholesterol synthesis, influx, transport, catabolism and efflux (MVK, LDLR, NPC1, CYP46A1 and ABCA1, respectively), and shown to be influenced by TSA treatment. SH-SY5Y cells were pre-treated with 3 µg/ml U18666A for 6 h, and subsequently with or without 250 nM TSA for 16 h. mRNA levels were detected by qPCR (Fig. 4.4).

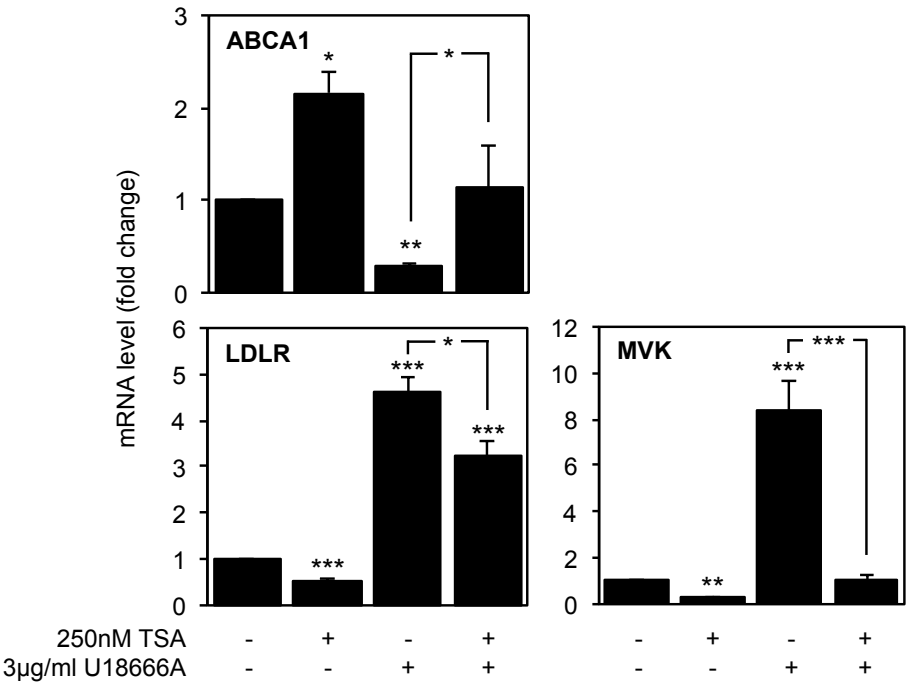

Figure 4.4. TSA treatment partially reverts the effect of U18666A on the expression of genes involved in cholesterol synthesis, uptake and efflux. SH-SY5Y neuroblastoma cells were pre-treated with 3 µg/ml U18666A for 6 h and with or without 250 nM TSA for 16 h. mRNA transcript levels of MVK, LDLR and ABCA1 were analyzed by qPCR. Values were normalized to the internal standard β-actin and are expressed as fold change relative to untreated cells. Data represent means ± SEM from at least three individual experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Since CYP46A1 and NPC1 mRNA levels were not affect by U18666A treatment (data not shown), we failed to observe if TSA could counteract the cholesterol accumulation induced by U18666A, by modulating the expression of these genes. Nevertheless, our results show that inhibition of intracellular cholesterol traffic induces a significant increase in the expression levels of MVK and LDLR to approximately 8- and 4.5-fold of control levels, respectively (ANOVA F = 33.799, df = 3, $p < 0.001$, Tukey HSD for unequal N $p < 0.001$ and ANOVA F = 138.349, df = 3, $p < 0.001$, Tukey HSD for unequal N $p < 0.001$). Interestingly, TSA can correct the effect of U18666a treatment on MVK expression levels, decreasing mRNA levels to control values. Moreover, TSA can also significantly revert the increase on LDLR mRNA levels induced by U18666a treatment (Tukey HSD $p < 0.05$). Interestingly, inhibition of intracellular cholesterol traffic significantly down-regulated ABCA1 to 30% of the control values (ANOVA F =

13.599, df = 3, $p < 0.001$; Tukey HSD for unequal N $p < 0.01$). TSA treatment can also correct this decrease to control levels. Overall, these results clearly demonstrate that TSA partially reverts the effect of intracellular cholesterol accumulation in the expression levels of genes involved in cholesterol homeostasis.

To assess if TSA was able to revert the phenotype of intracellular cholesterol accumulation induced by U18666A in a neuronal derived cell line such as the SH-SY5Y, we performed immunocytochemistry experiments in cells treated with 1 μg/ml U18666A for 24 h, and subsequently with 250 nM TSA or vehicle for 16 h (Fig. 4.5). Cholesterol accumulation was evaluated by filipin staining and its association with late endosomes/lysosomes was assessed by co-localization with the lysosomal marker LAMP-2. Since our goal was to evaluate the effect of TSA on the improvement of the U18666A-induced phenotype, we pre-treated cells with this compound for 24 h, since a significant cholesterol accumulation was already observed at this time point (Fig. 4.3A). The culture medium was then removed and new medium with TSA or vehicle was added to cells. Fluorescence quantification after filipin staining indicated that no significant changes in cellular cholesterol loading were detected after TSA treatment (Fig. 4.5B), which is in accordance with the results obtained for total cholesterol levels (Fig. 4.2). However, we observed a decrease of approximately 40% in the co-localization of cholesterol and the LAMP-2 marker after TSA treatment (Fig. 4.5C), which suggests that this HDACi is initially inducing the release of cholesterol from late endosomes/lysosomes and therefore promoting cholesterol redistribution in the cell.

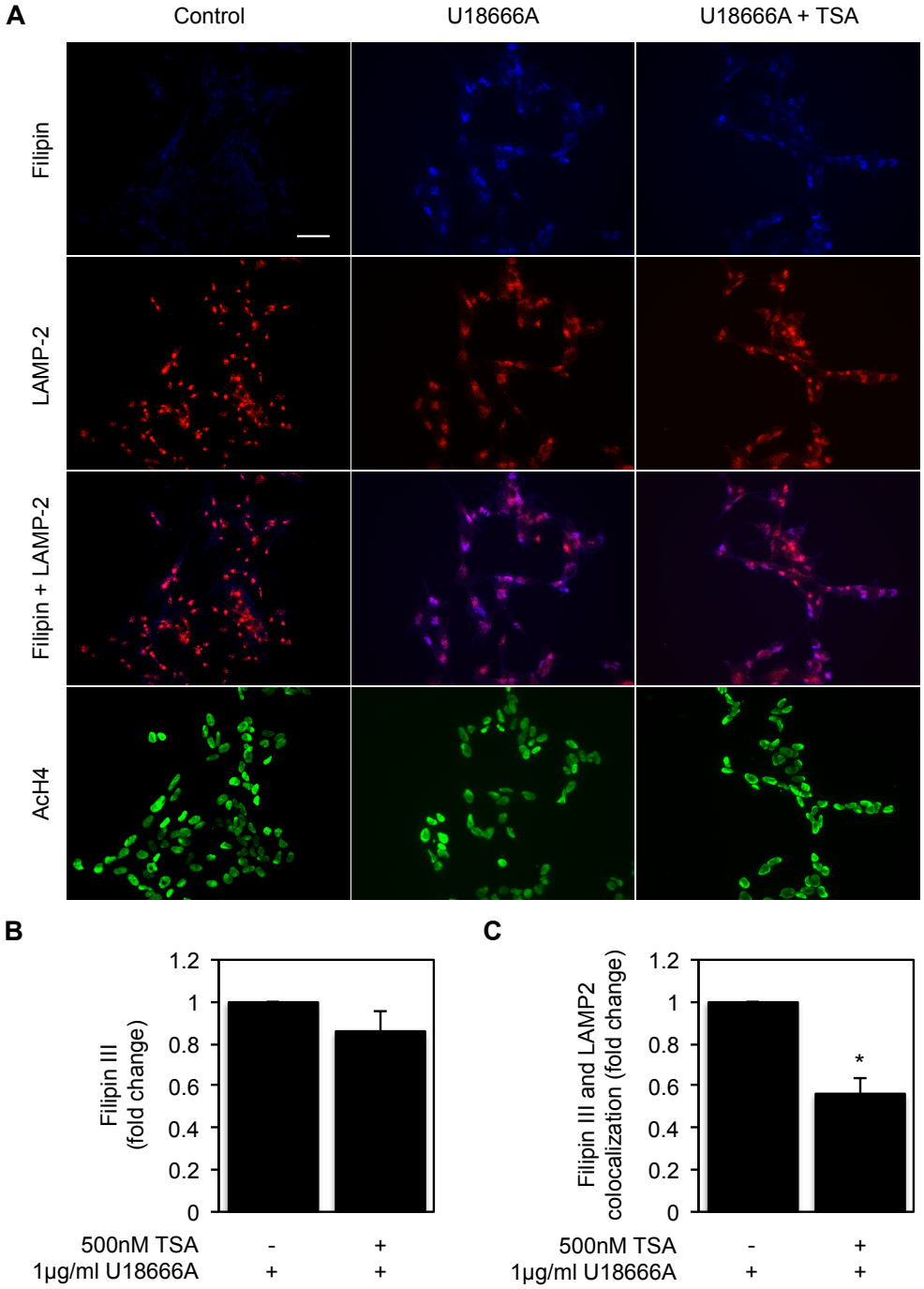

Figure 4.5. TSA treatment partially reverts the U18666A-induced phenotype by promoting cholesterol redistribution in neuroblastoma cells. A) Filipin III, lysosome-associated membrane protein 2 (LAMP-2) and acetyl-histone 4 (AcH4) immunofluorescence staining of SH-SY5Y cells pre-treated with 1 µg/ml U18666A for 24 h and with or without 500 nM TSA for 16 h. After the 24 h treatment with U18666A, the medium was removed and replaced with new medium without U18666A, and with or without TSA. Co-localization images of filipin III and LAMP-2 immunostaining are also presented. The results shown are representative of those obtained in at least three independent experiments (*scale bar* = 40 µm). Quantification of filipin III fluorescence (B) and filipin III and LAMP-2 co-localization (C) was performed, the values were normalized to total cell number assessed by AcH4 immunosatining and are expressed as fold change relative to U18666A treated cells. Data represent means ± SEM of at least three independent experiments (* $p < 0.05$).

4.5. Discussion

CYP46A1 is a key enzyme in brain cholesterol homeostasis. This neuronal-specific cytochrome P450 mediates the conversion of cholesterol into 24OHC (Lund *et al.* 1999), which represents the major pathway of cholesterol turnover in the CNS (Bjorkhem *et al.* 1998, Xie *et al.* 2003). As we have demonstrated in our previous studies that HDACi were able to significantly up-regulate *CYP46A1* transcription levels (Nunes *et al.* 2010), we hypothesized that these compounds would probably be able to control neuronal cholesterol levels, due to the importance of this enzyme in cholesterol homeostasis. That idea led us to investigate if and how HDAC inhibition was affecting the transcription of other genes involved in cholesterol metabolism. The studies described here clearly demonstrate that, in fact, TSA has the ability to decrease total cholesterol content of neuroblastoma cells by up-regulating genes responsible for cholesterol catabolism (CYP46A1) (Nunes *et al.* 2010) and efflux (ABCA1), and down-regulating genes responsible for cholesterol synthesis (HMGCR, HMGCS and MVK) and uptake (LDLR). Amongst these genes, CYP46A1 is the only one that has already been described to respond to HDACi treatment through hyperacetylation of the promoter in neuronal derived cells (Nunes *et al.* 2010). HMGCR, HMGCS and MVK were described to be up-regulated by HDACi (Villagra *et al.* 2007), along with ABCA1 (Xu *et al.* 2011), although both studies were performed in non-neuronal cell lines. By directly correlating TSA-mediated modulation of the transcription of genes involved in cholesterol metabolism to changes in total cellular cholesterol levels, our results suggest that the TSA effect is probably due to promoter acetylation/deacetylation-dependent mechanisms.

It has recently been reported by two different studies that HDACi treatment can reverse the phenotype of cholesterol accumulation and correct transport defects in human NPC fibroblasts (Munkacsi *et al.* 2011, Pipalia *et al.* 2011). HDACi effect was suggested to depend on restoration of the expression of the majority of HDACs that were up-regulated in NPC cells (Munkacsi *et al.* 2011), or be associated with an increased stabilization of NPC1 protein levels (Pipalia *et al.* 2011). Nevertheless, the exact mechanisms remain to be determined. In addition, HDACs play important roles in several neurological functions (Haggarty & Tsai 2011), and HDACi have been described to be neuroprotective in various neurodegenerative models (Chuang *et al.* 2009). Indeed, overexpression of neuronal specific HDAC2 decreases dendritic spine density, synapse

number, synaptic plasticity and memory formation, which are reverted by HDAC inhibition (Guan *et al.* 2009). Interestingly, cholesterol is also essential for synaptogenesis (Mauch *et al.* 2001, Goritz *et al.* 2005) and dendrite outgrowth (Fan *et al.* 2002). Some studies have already identified specific HDACs involved in cholesterol and lipid metabolism. HDAC3 has been described to down-regulate cholesterol synthesis in HeLa cells, through the repression of lanosterol synthase expression, an enzyme of the biosynthetic cholesterol pathway (Villagra *et al.* 2007). The studies of Munkacsi and co-workers (Munkacsi *et al.* 2011) identified a significant up-regulation of HDAC4 in fibroblasts of NPC patients, along with HDAC6 and 11, which was markedly corrected by HDAC inhibition. Several HDAC candidates could mediate the TSA effect; however, their identification can only be achieved by specific chemical inhibition concomitantly with efficient knockdown by siRNA transfection.

Although neurodegeneration is the fatal cause in NPC patients, the majority of experimental approaches are performed in fibroblasts. Therefore, we evaluated if TSA could ameliorate the phenotype induced by pathological cholesterol accumulation in human neuronal cells. We used the chemical compound U18666A to mimic the accumulation of cholesterol in late-endosomal/lysosomal compartments observed in cells from NPC patients (Cenedella 2009). Neuroblastoma SH-SY5Y cells were treated with U18666A, which increased total cholesterol levels. This was associated with an increase in the transcription of genes involved in cholesterol synthesis (MVK) and uptake (LDLR), and a decrease in cholesterol efflux (ABCA1). These changes induced by U18666A are also characteristic of NPC disease. The NPC phenotype of unesterified cholesterol accumulation in late endocytic organelles, as a result of defective cholesterol transport due to mutations in NPC proteins, is also characterized by impaired cholesterol esterification (Pentchev *et al.* 1985) and inefficient suppression of cholesterol synthesis and LDLR activity (Liscum & Faust 1987). Interestingly, we observed that TSA partially revert the changes induced by U18666A, through the correction of MVK, LDLR and ABCA1 gene transcription. The modulation of LDLR is of particular importance since lipoprotein uptake through the LDLR-mediated endocytosis is the major pathway leading to accumulation of unesterified cholesterol in NPC cells (Xie *et al.* 1999). In the context of the CNS, astrocytes cooperate with neurons in the supply of cholesterol in the form of ApoE containing lipoproteins that subsequently are taken up by neurons through the LDLR (Pfrieger 2003). Interestingly, restoring the Npc1 in Npc $^{-/-}$ mice only in astrocytes

enhanced survival and decreased neuronal storage of cholesterol and neuronal degeneration (Zhang *et al.* 2008).

In addition to the end-point effects of TSA treatment, early time-points of treatment showed a reduction in the amount of free cholesterol associated with late endosomes/lysosomes. This evidence suggests that alterations in the intracellular cholesterol transport induced by HDAC inhibition are facilitating cholesterol removal from the cell. Although we demonstrated the TSA induces NPC1 expression, a protein crucial for intracellular movement of cholesterol out of the late-endosomal system and into the cytosol and other organelles, U18666A has no effect on the transcription of this gene, indicating that release of cholesterol from endosomes was not dependent on an increase in NPC1expression. Nevertheless, improvement of cholesterol movement from late-endosomes to the plasma membrane and to the ER could be sensing the cells to cholesterol leading to a down-regulation of synthesis and uptake, by the SREBP mediated pathway (Goldstein *et al.* 2006). Therefore, mechanisms independent of promoter acetylation/deacetylation must also be taken into account.

Overall, this study describes how HDACi modulate cholesterol metabolism at the transcription level in neuroblastoma cells, thus reinforcing the idea of inhibiting HDACs as an effective therapeutic avenue to target neurodegenerative conditions that lead to cholesterol accumulation.

4.6. Acknowledgements

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CHAPTER 5

CONCLUDING REMARKS

The original work presented in this thesis identify histone deacetylation as an epigenetic mechanism involved in the regulation of *CYP46A1* gene transcription, and characterize TSA as an epigenetic modifier that can regulate neuronal cholesterol metabolism.

In Chapter 2 and Chapter 3 we identify HDACi, with a special focus on TSA, as potent activators of *CYP46A1* transcription, and characterize, for the first time, the molecular mechanisms and players that trigger this effect. The regulatory mechanisms deciphered by our studies are schematically represented in Fig. 5.1.

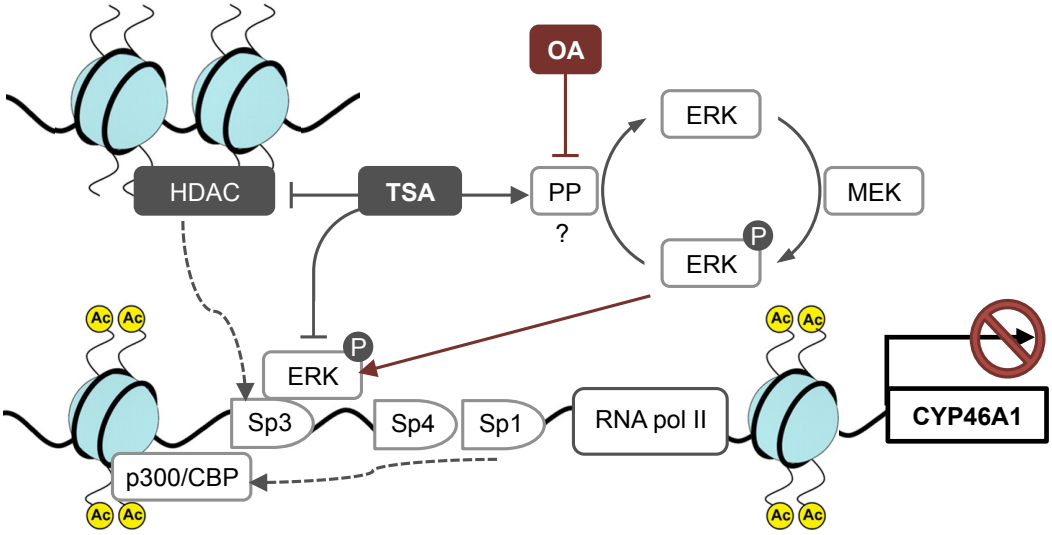

Figure 5.1. Mechanism of *CYP46A1* activation by the HDACi TSA. *CYP46A1* transcription is significantly increased after treatment with the HDACi TSA, reflected by the recruitment of RNA pol II. The increase in histone acetylation is mediated by the recruitment of p300/CBP to the proximal promoter concomitantly with the detachment of HDAC1 and HDAC2, in a process that depends on Sp1 and Sp4 but is favored by decreased binding of Sp3 to the promoter. Moreover, TSA also decreases the activation of ERK1/2 and Sp3, which appears to be crucial for the overall TSA effect. Impairment of Sp3 dephosphorylation, by inhibiting protein phosphatase activity with okadaic acid almost completely abolishes the TSA effect.

In our initial studies we observed a significant increase in *CYP46A1* promoter activity and mRNA levels after HDACi treatment. Interestingly, in the first study (Chapter 2) we were able to confirm for the first time, by chromatin immunoprecipitation, that Sp transcription factors, previously suggested to be essential for *CYP46A1* basal expression (Milagre *et al.* 2008), bind to the proximal promoter of this gene. Moreover, we have shown that Sp protein binding to the GC-rich region at the proximal promoter is

also critical for the TSA response. Our results showed that the transient histone hyperacetylation at the CYP46A1 promoter after TSA treatment was caused by a shift in the HDAC/HAT equilibrium, promoted by alterations in the balance between the different Sp family members, that ultimately culminated in the recruitment of RNA pol II and accumulation of CYP46A1 transcripts. These results suggest that it is possible to modulate CYP46A1 in the brain by pharmacological means, which is extremely relevant in light of the results of Hudry and co-workers (Hudry *et al.* 2010), demonstrating that CYP46A1 overexpression significantly decreased Aβ pathology in mouse models of AD. Indeed, treatment with HDACi has been suggested as a future therapeutic approach to treat this disease. It has been reported that VA, an HDACi used as anticonvulsant and mood-stabilizing agent, inhibits Aβ production, neuritic plaque formation, and behavioral deficits in Alzheimer's disease mouse models (Qing *et al.* 2008). In addition, 4-phenylbutyrate decreases tau pathology, reverts spatial memory deficits in Tg2576 mouse model of AD, without affecting amyloid β levels (Ricobaraza *et al.* 2009), while rescuing dendritic spine loss associated with memory deficits in the same animal model (Ricobaraza *et al.* 2012). More recently, the cognitive decline observed in a mouse model of AD was attributed to transcriptional impairment related to increased expression of HDAC2, also observed in post-mortem brain samples of AD patients, but reversion of the HDAC2 up-regulation restored synaptic plasticity (Graff *et al.* 2012). Altogether, these studies suggest that HDAC inhibition could improve several aspects of AD, which emphasizes the importance of better understanding the molecular mechanisms involved in HDAC inhibition.

With that in mind, in subsequent studies (Chapter 3) we further detailed the mechanism of TSA-mediated *CYP46A1* activation, particularly the participation of the MEK/ERK signaling pathway. While specific silencing/inhibition of this pathway clearly potentiated the TSA effect, inhibition of PPs (through OA treatment) had the opposite outcome, suggesting that a critical dephosphorylation event could be mediating the transcriptional changes observed after TSA treatment. Indeed, TSA induced a decrease in ERK1/2 phosphorylation that was reverted by OA treatment, concomitantly with a drastic reduction of CYP46A1 mRNA levels, although no alterations in the histone acetylation levels of the *CYP46A1* promoter were observed. Decreased activation of ERK1/2 upon TSA treatment was found to be responsible for a reduction of Sp3 binding activity possibly by regulating its phosphorylation levels, which was suggested by the decreased

association of Sp3 and ERK1/2 in *CYP46A1* proximal promoter. Interestingly, we had previously demonstrated that Sp3 and HDACs detachment from the *CYP46A1* promoter after treatment of neuroblastoma cells with the demethylating agent 5-aza-2-deoxicytidine, not only induced *CYP46A1* gene transcription, but also synergistically enhanced TSA-mediated derepression of this gene (Milagre *et al.* 2010). These studies clearly identify Sp3 as a transcriptional repressor of *CYP46A1* gene transcription in response to epigenetic drugs. Interestingly, during differentiation of NT2 cells into *post-mitotic* neurons, an increase in CYP46A1 mRNA and protein levels was associated with a decrease in the total amount of Sp1 protein and also a detachment of this transcription factor from the *CYP46A1* promoter (Milagre *et al.* 2012a, Milagre *et al.* 2012b). Our results are therefore, in accordance with the previous suggestion that Sp1 may be more involved in the control of basal transcription, whereas Sp3 mediates inducible transcription (Ward & Samuel 2003). However, since the effects of inhibiting PPs and MEK-ERK pathway were replicated in the basal *CYP46A1* promoter activity and mRNA levels, it would be important to evaluate if Sp3 is also mediating these effects.

In the case of the effect of OA pre-treatment on TSA activation of *CYP46A1* expression, one hypothesis is that phosphorylated Sp3 can recruit co-repressor complexes with no HDAC/HAT activity, since histone acetylation appears to be unaffected at least at the time points analyzed. Another open question is the identification of the PP responsible for the abrupt inhibition of both *CYP46A1* activation by TSA and basal activity. Although OA is widely used to specifically inhibit PP2A, the fact is that both PP4 and PP5 are inhibited by low concentrations of this compound, which makes the interpretation of the chemical inhibition data difficult. Although fostriecin, another PP inhibitor, could eventually be used to differentiate between PP2A/PP4 and PP1/PP5 (Honkanen & Golden 2002), specific silencing will be the ultimate confirmation. Nevertheless, PP5 is a plausible candidate, since it is highly expressed in the brain, has been described to dephosphorylate tau protein and to present reduced activity in AD neocortex (Liu *et al.* 2005), whilst having a protective effect in neurons against Aβ toxicity (Sanchez-Ortiz *et al.* 2009). Furthermore, PP5 seems to regulate the Raf-MEK-ERK pathway (von Kriegsheim *et al.* 2006, Mkaddem *et al.* 2009). Since PP inhibition also decreases basal *CYP46A1* expression, it could be the case that the observed reduced activity of PPs in AD contributes not only to tau hyperphosphorylation pathology but also to deregulation of

neuronal cholesterol metabolism, which is associated with Aβ pathology (Simons *et al.* 1998, Fassbender *et al.* 2001, Kojro *et al.* 2001).

Modulation of different signaling pathways by HDACi has been described in diverse contexts (Witt *et al.* 2000, Rivero & Adunyah 1998, Chen *et al.* 2005), and the fact that TSA promotes ERK1/2 dephosphorylation in our cellular model raises the question whether this event is affecting the activity of the other players involved. Both CBP and p300 are phosphorylated in a ERK1/2-dependent manner (Liu *et al.* 1999, Gusterson *et al.* 2002), which promotes acetyltransferase activity and transcriptional complex formation. Moreover, HDAC1 and HDAC2 activity is also increased after phosphorylation by casein kinase 2 (Pflum *et al.* 2001, Tsai & Seto 2002), and although MAPK do not appear to affect at least HDAC1 phosphorylation levels (Cai *et al.* 2001), it is not known if TSA can modulate HDAC/HAT activity by interfering with other signaling cascades. Indeed, preliminary unpublished results suggest the participation of AMPK in the transcriptional changes induced by TSA, in an opposite way of that of the MEK-ERK signaling pathway: inhibition of AMPK with compound C drastically impairs *CYP46A1* derepression by TSA, whereas activation with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) potentiates the TSA effect. Although the participation of this signaling pathway remains to be clarified it is interesting to notice that in cardiac fibroblasts an inhibitory cross talk between ERK and AMPK was described (Du *et al.* 2008).

Interestingly, depletion of membrane cholesterol was shown to increase p-ERK levels in cellular caveolae and cytosolic fractions (Furuchi & Anderson 1998). Regulation of ERK phosphorylation by cholesterol was described to be mediated by an oligomer complex composed of an oxysterol-binding protein (OSBP) associated with two phosphatses, the tyrosine phosphatase HePTP and the serine/threonine PP2A, and cholesterol, with dual specific phosphatase activity for p-ERK (Wang *et al.* 2003, Wang *et al.* 2005). Cholesterol controls the oligomer assembling by interacting with OSBP: depletion of cholesterol or treatment with 25OHC promotes the complex disassemble, which inhibits phosphatase activity, resulting in increased levels of p-ERK. The identification of MEK-ERK pathway in the regulation of *CYP46A1* transcription raises an interesting hypothesis that in the presence of high cholesterol levels, an increase in CYP46A1 activity could lead to an increase in cholesterol removal and in 24OHC levels, and consequently in ERK activation and down-regulation of *CYP46A1* gene transcription.

Due to the ability of HDACi to modulate *CYP46A1* expression, and therefore to affect cholesterol catabolism in the brain (Bjorkhem *et al.* 1998), we further explored how these compounds (TSA) could affect the expression of other genes involved in cholesterol metabolism in neuronal cells at the transcriptional level (Chapter 4). Although it had been already been suggested that HDACi could modulate cholesterol metabolism (Chittur *et al.* 2008, Villagra *et al.* 2007, Xu *et al.* 2011), we were able to demonstrate that TSA treatment significantly decreases total cholesterol content in neuroblastoma cells, through the transcriptional modulation of key genes in cholesterol metabolism, namely down-regulation of synthesis (HMGCR, HMGCS and MVK) and uptake (LDLR), and up-regulation of cholesterol efflux (ABCA1) and catabolism (CYP46A1). Given this general transcriptional effect towards decreased cholesterol load, it is essential to understand if TSA is modulating transcription through changes in histone acetylation levels at the promoters of these genes. Moreover, the use of more specific HDACi concomitantly with specific HDAC silencing will elucidate which HDAC(s) are involved in the control of neuronal cholesterol metabolism. Since some of the effects of HDACi in cancer cells cannot be recapitulated in nontransformed cells (Dokmanovic & Marks 2005), it will be extremely important to characterize how primary cultures of rat cortical neurons respond to TSA treatment.

One of the interesting effects of TSA treatment is that cholesterol synthesis and uptake are simultaneously decreased, implying a bypass of the classic transcriptional regulation by SREBPs in response to lower levels of cholesterol. Preliminary results from our group appear to indicate that, despite no differences in SREBP2 mRNA levels were observed upon TSA treatment, active/cleaved SREBP2 protein levels are decreased, which could explain the transcriptional consequences observed. Therefore, it would be important to assess how TSA is modulating SREBP2 proteolytic cleavage. Interestingly, transactivation activity of these transcription factors has also been described to be regulated by several signaling pathways, including the MEK-ERK pathway (Kotzka *et al.* 2000, Kotzka *et al.* 2004). Evaluating the phosphorylation levels of SREBP proteins after TSA treatment and identification of specific signaling pathway(s) modulated by TSA in neuronal cells is indispensable for understanding its role as a cholesterol-lowering molecule.

When comparing the TSA effect on the induction of *CYP46A1* gene transcription by TSA with the remaining genes analyzed the differences are drastic. Although

significant alterations in the mRNA levels of MVK (reduction of 70%) or ABCA1 (2-fold increase) were determined, TSA induces *CYP46A1* transcription by 300-fold. Therefore, another question can be raised: is the observed effect in the transcription of cholesterol metabolism key enzymes after TSA treatment a consequence of changes in cellular cholesterol content induced by *CYP46A1* activation? Ongoing work in our group consistently indicates that transient overexpression of the human CYP46A1 protein in primary cultures of rat cortical neurons significantly decreases membrane cholesterol content. We are currently evaluating the transcriptional profile of the genes previously confirmed to be modulated by TSA treatment, and assessing SREBP2 cleavage and p-ERK levels, in neuroblastoma cells, after CYP46A1 transient overexpression/24OHC treatment.

Our results also demonstrate for the first time that treatment with an HDACi can reverse the increase in cholesterol levels and the transcriptional changes induced by U18666A, a compound that mimics the NPC phenotype in neuronal cells. Moreover, we also demonstrated that TSA is able to improve cholesterol movement from late endosomes to the plasma membrane and to the ER after cholesterol accumulation by U18666A treatment. In this way, TSA treatment can help the cell to do the correct sensing of intracellular cholesterol levels at the ER, leading to a down-regulation of synthesis and uptake, by the SREBP mediated pathway (Goldstein *et al.* 2006), which may help to restore intracellular cholesterol homeostasis.

Overall, the work presented in this thesis contributed to the elucidation of the molecular mechanisms governing *CYP46A1* transcriptional regulation, particularly the identification and characterization of HDAC inhibition as a mechanism of *CYP46A1* activation. Moreover, by describing the cholesterol-lowering effect of TSA treatment in neuronal cells, through the modulation of the expression levels of key genes involved in cholesterol metabolism, including up-regulation of *CYP46A1* transcription and consequently cholesterol catabolism, these studies further clarified the potential therapeutic approach of HDACi in neurodegenerative disorders with altered cholesterol metabolism.

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